NUCLEAR RECEPTORS ASSOCIATED WITH LIPID METABOLISM AND THEIR ROLE IN THE SIDE EFFECTS ASSOCIATED WITH ANTIRETROVIRAL THERAPY

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

By
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DECLARATION

This thesis is the result of my own work. The material contained within the thesis has not been presented, either wholly or in part, for any other degree or qualification.

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ABSTRACT

Anti-retroviral therapy comprises a combination of three or more drugs from at least two classes. Following long term therapy some patients experience metabolic side-effects including abnormal fat distribution, dyslipidaemia, lipoatrophy, hyperlipidaemia, impaired lipid and glucose metabolism. Metabolic nuclear receptors may be implicated in some of these deleterious side effects.

RTV is widely implicated in the deleterious metabolic side effects of ARVs, whilst ATV has been shown to have a better lipid profile than RTV. Previous studies had not determined whether this was due to differences in intra-cellular accumulation of the two drugs. Cellular accumulation of ATV was determined to be 2 fold greater than that of RTV in the hepatic cell line, Huh7. This confirmed that the metabolic disruption and the deleterious side effects associated with RTV were not due to its higher intracellular accumulation.

LXRα was significantly up-regulated by RTV, and it was determined to be a regulator of key downstream target gene expression, including SREBP-1c, PPARα, ApoA-I, VLDLR, ApoC-II and ApoC-III. Inhibition of LXRα by 22(S)-hydroxycholesterol and by siRNA knockdown of LXRα was used to confirm these findings. Biological knockdown of LXRα by siRNA resulted in down-regulation of LXRα gene expression in the presence of RTV, which could provide a potential future therapeutic mechanism for inhibition of LXRα *in vivo*.

LXRα was involved in the regulation of gene expression of bile acid and cholesterol transporters including: ABCA1, ABCB2, ABCG1, ABCG4, ABCG5, ABCG8 and the glucose transporter GLUT4, which were up-regulated by RTV. LXRα was involved in the regulation of ABCB11 and ABCC2 but data suggested that LXRα was not the master regulator. Gene expression of these metabolic transporters differs in response to RTV and ATV. The actions of RTV can be blocked through modulation of LXRα gene expression. LXRα was also involved with the regulation of ABCB1 and ABCC1 gene expression.

A polymorphism in LXRα, rs2279238C/T, could influence the risk of HADL development in Caucasian HIV+ populations. The results indicate further investigation would be required in a larger HIV cohort to substantiate this association.

Elevated LDL-c levels were observed in HIV+ Caucasians homozygous GG genotype for the ApoB rs7575840GT SNP and with at least one copy of the A allele for the ABCA1 rs2230806G/A SNP. The C allele in the LDLR rs1529729C/T SNP was associated with elevated total cholesterol, LDL-c and TG levels. Elevated total cholesterol and increased LDL-c levels were observed when this SNP was combined with the GG genotype for ApoB rs7575840GT and the C allele for the LDLR SNP rs2228671C/T. Decreased HDL-c was observed in HIV+ patients with the T allele of the SCARB1 SNP rs2278986C/T when associated with a combination of demographic factors and the following SNPs: ABCA1 rs2230806G/A, A allele; LDLR rs1529729C/T, C allele; ABCG1 rs1893590A/C, C allele; ABCG8 rs6544713C/T, T allele; and ApoB rs693A/G, G allele.

In summary the data in this thesis indicates LXRα is a regulator of metabolic target genes involved in cholesterol homeostasis, bile acid metabolism and the response to RTV in a hepatic cell line. The role of RTV as an agonist of LXRα was confirmed. The contrasting lipid profiles of RTV and ATV are not due to higher intracellular concentration of the former. This thesis has also identified a potential therapeutic use for LXRα modulation. SNPs in LXRα and key cholesterol transporters have correlated with metabolic complications in HIV+ cohorts which merit further investigation in larger patient cohorts to validate the associations.

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LIST OF PUBLICATIONS

Publications and Posters

-2008. 3rd Liverpool-Bristol Symposium, Liverpool, UK. Defining the Profile of Activation for Metabolic Nuclear Receptors by Antiretroviral Drugs. *Poster Presentation.*

-2009. University of Liverpool Poster Day, Liverpool, UK. Defining the role of the Metabolic Nuclear Receptors in the Complications Associated with Long Term HIV Therapy. *Poster Presentation.*

-2009. MRC Centre for Drug Safety Science Inaugural Symposium with the MHRA and ABPI, Liverpool, UK. The role of the Metabolic Nuclear Receptors in the Complications Associated with Antiretroviral Drugs. *Poster Presentation.*

ABBREVIATIONS

ABC	Adenosine tri-phosphate binding cassette transport family
AF-1	Activation function 1 domain
AF-2	Activation function 2 domain
AIDS	Acquired immune deficiency syndrome
ApoA-I	Apolipoprotein-A1
ApoB	Apolipoprotein B
ApoC-II	Apolipoprotein C-II
ApoC-III	Apolipoprotein C-III
ARVs	Antiretroviral drugs
ATP	Adenosine tri-phosphate
ATV	Atazanavir
β2M	βeta-2-Microglobulin
BSEP	Bile salt export pump
$^{\circ}C$	Degrees Celsius
CAR	Constitutive androstane receptor
CCR5	Chemokine Co-Receptor 5
CD4	Cluster of differentiation 4
$CD4^{+}$	CD4 positive
$CD8^{+}$	CD8 positive
cDNA	Complementary deoxyribonucleic acid
CERP	Cholesterol efflux regulatory protein
C_T	Cycle Threshold
CYP	Cytochrome P450 isoenzymes
DBD	Deoxyribonucleic acid binding domain
dl	Decilitre
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DR	Direct repeat
dsDNA	Double stranded deoxyribonucleic acid
EIs	Entry inhibitors (Fusion inhibitors)
FAs	Fatty acids
FAS	Fatty acid synthase
FBS	Foetal bovine serum
FCS	Foetal calf serum
FDA	Food and Drug Administration
FFAs	Free fatty acids
FIs	Fusion inhibitors
FXR	Farnesoid X activated receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT4	Facilitated glucose transporter 4

G6Pase	Glucose-6-phosphatase
GR	Glucocorticoid receptor
h	Hour(s)
HAART	Highly active antiretroviral therapy
HADL	Human immunodeficiency virus/highly active antiretroviral therapy associated dyslipidaemic lipodystrophy
HBSS	Hanks balanced salt solution
HCV	Hepatitis C virus
HDL	High density lipoprotein
HDL-c	High density lipoprotein cholesterol
HIV	Human immunodeficiency virus
HIV+	Human immunodeficiency virus positive
HNF4α	Hepatic nuclear receptor 4 alpha
HRE	Hormone response element
HSP70	Heat shock protein 70
IBABP	Intestinal bile acid binding protein
IIs	Integrase inhibitors
IR	Inverted repeat
LBD	Ligand binding domain
LCA	Lithocholic acid
LCAT	Lecithin cholesterol acyltransferase
LDL	Low density lipoprotein
LDL-c	Low density lipoprotein cholesterol
L-GK	Liver glucokinase
LHPG	Liverpool HIV Pharmacology Group
LPL	Lipoprotein lipase
LRH-1	Liver receptor homologue-1
LXRα	Liver oxysterol activated receptor alpha
MDR1	Multidrug resistance transporter 1
mg	Milligrams
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
MRP1	Multidrug resistant protein 1
MRP2	Multidrug resistant protein 2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ng	nanogram
NIH	National Institute of Public Health (USA)
nM	nanomolar
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRs	Nuclear receptors
NRTIs	Nucleoside/nucleotide reverse transcriptase inhibitors
NSAIDs	Non-steroidal anti-inflammatory drugs

PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
P-gp	P-glycoprotein
PI	Protease inhibitor
PLTP	Phospholipid transfer protein
PPARα	Peroxisome proliferator activated receptor alpha
PPARγ	Peroxisome proliferator activated receptor gamma
PPARδ	Peroxisome proliferator activated receptor delta
PPRE	Peroxisome proliferator response element
PUFAs	Polyunsaturated fatty acids
PXR	Pregnane xenobiotic activated receptor
RE	Response element
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT PCR	Real time polymerase chain reaction
RTV	Ritonavir
RXRα	Retinoid X activated receptor alpha, 9-*cis* retinoic acid receptor alpha
s	Seconds
SGBS	Simpson Golabi Behmel Syndrome adipocyte cell line
siRNA	Small interfering ribonucleic acid
SHP-1	Small heterodimer partner-1
SLC2A4	Solute carrier family 2 member A4
SLC10A1	Solute carrier family 10 member A1
SNP	Single nucleotide polymorphism
SQV	Saquinavir
SREB	Sterol regulatory binding protein
SREBP-1c	Sterol regulatory binding protein 1c
SULT2A1	Sulphotransferase, cytosolic 2 family, polypeptide A1
TGs	Triglycerides
UCP	Uncoupling protein
μg	Microgram
UGT2B4	Uridine diphosphate glucoronosyl transferase 2 family, polypeptide B4
μl	Microlitre
μM	Micromolar
VDR	Vitamin D receptor
VLDL	Very low density lipoprotein
VLDLR	Very low density lipoprotein receptor
XREM	Xenobiotic responsive enhancer module

CHAPTER 1

GENERAL INTRODUCTION

1. General Introduction

1.1 History of HIV and HIV Life Cycle

Human immunodeficiency virus (HIV) is a retrovirus which uses the cells of the human immune system to survive and reproduce. There are two main types of HIV, HIV-1 and HIV-2, the former is the more virulent whereas the latter is confined mainly to West African populations and sexual partners. Recognised sub-types of both HIV-1 and HIV-2 are known to exist. By the end of 2009, it was estimated that around 33.3 million people worldwide were living with HIV/AIDS (acquired immunodeficiency syndrome), there were around 2.6 million new HIV infections and approximately 1.8 million people died from AIDS during the same year (UNAIDS, 2010). There is no current cure or vaccine for HIV or AIDS. Current drug therapy aims to reduce the viral load in plasma to below detectable levels and to maintain this for as long as possible. Antiretroviral drugs dramatically increase life expectancy but there are side effects associated with long-term use.

HIV infection of cells commences when the HIV virion (HIV virus is known as a virion when outside the cell), which is surrounded by a glycoprotein envelope, binds to both a CD4 receptor and a chemokine co-receptor on the surface of the host's immune cells. These include T-helper cells, monocytes, macrophages and microglial cells. Viral tropism refers to the cell types infected by HIV. Macrophage (M-tropic) strains of HIV-1 use the chemokine co-receptor CCR5 for entry and T-tropic isolates replicate in primary $CD4^+$ T cells as well as in macrophages and use the α-chemokine receptor, CXCR4, for entry. More than seven chemokine co-receptors act as co-receptors for HIV entry to cells, including CXCR4 and CCR5 (major co-

receptors *in vivo*). HIV variants which use CXCR4 or both CXCR4 and CCR5 (dual tropic) co-receptors are temporarily associated with accelerated $CD4^+$ T-cell depletion and rapid disease progression (Simmons *et al.*, 1996).

The key stages of the HIV Lifecycle are outlined in Figure 1.1 (Abbas *et al.*, 2003). The HIV virion envelope is a complex composed of a transmembrane glycoprotein (gp41) subunit and an external gp120 subunit. The virion envelope is expressed as a trimeric structure comprised of three gp120/gp41 pairs, which mediates a multistep process of fusion of the virion envelope with the membrane of the host cell. The first step involves the binding of the gp120 subunits to CD4 molecules, inducing a conformational change which promotes secondary gp120 binding to a chemokine co-receptor. Co-receptor binding causes a conformational change in gp41 exposing a hydrophobic region known as the fusion peptide, which inserts into the cell membrane enabling fusion of the viral and host cell membranes (Abbas *et al.*, 2003).

Once inside the cell, the virion uncoats and enzymes within the nucleoprotein complex are activated initiating the viral reproductive cycle. Reverse transcriptase transcribes the RNA genome of HIV into double stranded DNA, which enters the host cell nucleus with the viral enzyme integrase, and catalyses the integration of viral DNA into the host cell genome. This integrated HIV DNA is the provirus. Viral replication is initially repressed by host intracellular factors and by the $CD8^+$ cytotoxic T-cell mediated immune response. It can remain latent for long periods, during which the virus is highly active within the cell but new virus particles are not released into the bloodstream (Abbas *et al.*, 2003 ; Kim *et al.*, 2006a).

Activation of latently infected T-cells initiates viral replication. The proteins which form new HIV particles are assembled within the host cell through the translation of messenger RNA. These proteins are then spliced by viral protease into smaller sections which form the structure, enzymes and replication proteins of the new HIV particle. Once this assembly is complete, the new viral particle buds off the host cell and is released into the bloodstream resulting in the infection of more $CD4^+$ cells.

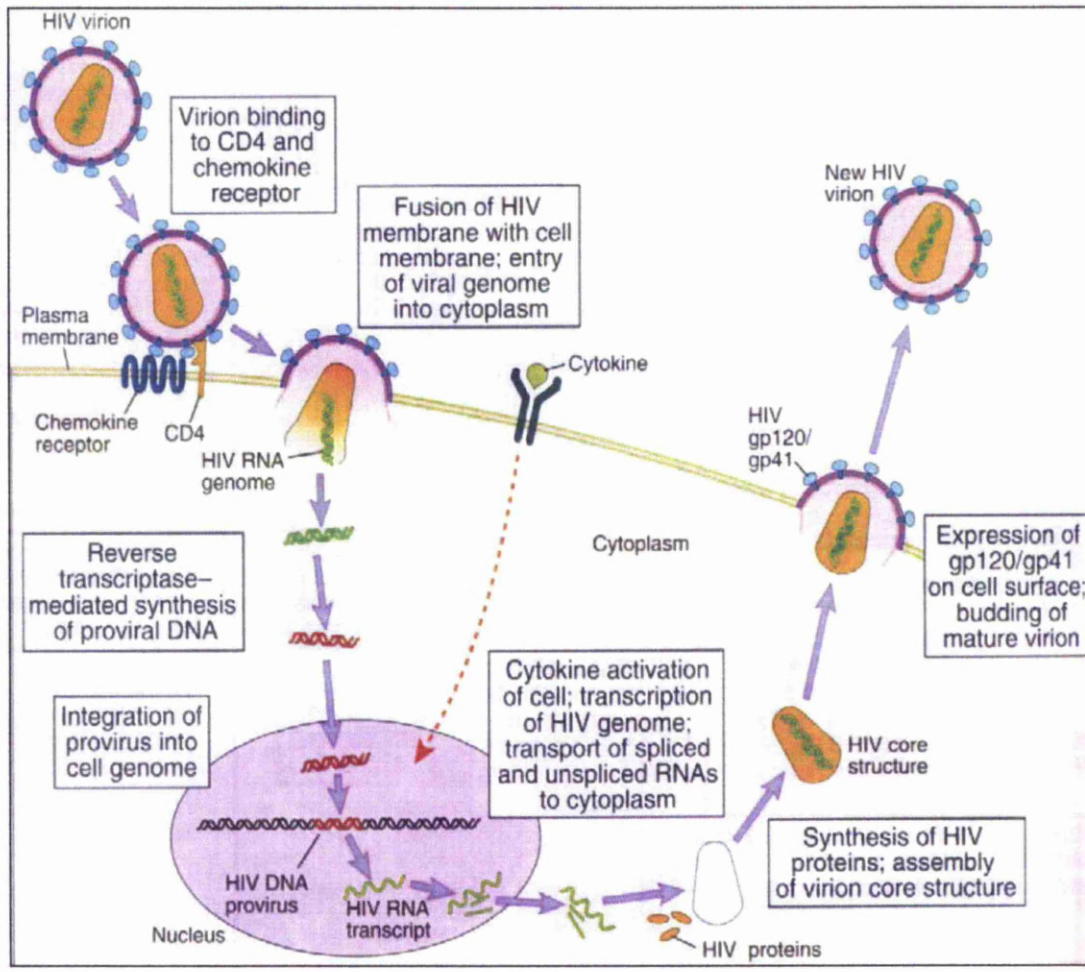


Figure 1.1: HIV life cycle – showing the sequential steps of the replication process. From Abbas and Lichtman: Cellular and Molecular Immunology, 2003; Figure 20-5.

1.2 Highly Active Anti-Retroviral Therapy (HAART)

Highly active antiretroviral therapy (HAART) comprises a combination of three or more antiretroviral drugs from at least two different classes, and has been around since the mid-1990s. HAART has significantly reduced the mortality and morbidity of HIV infection, giving a much transformed outlook to the infected (Falutz, 2007). It is a lifelong drugs regimen and with long-term therapy side-effects are common (Behrens *et al.*, 2000; Bonfanti *et al.*, 2000; Carr, 2000; Mallon, 2007). HAART can in many cases drive down the virus level in the body to levels undetectable by current assays. This, however, is not always successful and a significant number of individuals have high levels of HIV despite their potent drug regime, whilst others experience transient improvement before their viral load returns to pre-treatment levels (Cressey *et al.*, 2007; Owen *et al.*, 2008; Telenti *et al.*, 2008).

The major causes of treatment failure include drug resistance and non-compliance with the complex drug regimen, but individual pharmacokinetic variability can also play a key role (Cressey *et al.*, 2007). A significant obstacle to finding a cure is the ability of the virus to find sanctuary from the host's immune response in the brain, other body compartments and the lymphatic system. The virus can remain latent in sanctuary reservoirs for long periods prior to being triggered for replication by a fall in drug concentrations. Viral replication under therapy can positively select drug resistant strains leading to virologic failure.

Drug intolerance (side-effects), poor access to medical care, inadequate support networks and substance abuse all contribute to non-compliance. HIV-1 positive

(HIV+) individuals receiving antiretroviral therapy sometimes experience serious metabolic side-effects including lipoatrophy, gains in visceral fat, hyperlipidaemia and insulin resistance (Mallon, 2007; Mallon *et al.*, 2003). It is likely that numerous mechanisms contribute to these side-effects, although prolonged treatment with a HAART regimen is widely implicated (Behrens *et al.*, 2000; Dube *et al.*, 2006; Falutz, 2007; Mallon *et al.*, 2003).

Thirty-two antiretroviral drugs (ARVs) including combination therapies are currently approved by the Food and Drug Administration (FDA), in the United States (twenty-nine in Europe) which fall into four major classes: reverse transcriptase (RT) inhibitors (including nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs)); protease inhibitors (PIs); chemokine co-receptor antagonists (entry and fusion inhibitors (EIs)); and integrase inhibitors (IIs). Multidrug combinations incorporate more than one class into a single product.

PIs inhibit the action of the HIV viral enzyme protease. The protease enzyme cleaves the inert polyprotein product of translation into the smaller structural and functional proteins required for viral growth, infectivity and replication. NRTIs require intracellular conversion to the corresponding tri-phosphate nucleotide for activation. The active tri-phosphates then competitively inhibit viral reverse transcriptase causing termination of DNA chain elongation once incorporated. NNRTIs bind to the viral enzyme reverse transcriptase close to the catalytic site causing a conformational change which inactivates the enzyme.

Fusion inhibitors interfere with the entry of HIV-1 into cells by inhibiting the fusion of viral and cellular membranes. Enfuvirtide is the only licensed fusion inhibitor in clinical use. It acts by binding to the first heptad repeat (HR1) in the gp41 subunit of the viral envelope glycoprotein preventing the conformational changes required for the fusion of viral and cellular membranes. EIs block the chemokine co-receptors on the host cells to prevent the HIV virion binding. Maraviroc is the only licensed EI currently in clinical use. It acts by blocking CCR5 preventing HIV from binding to the receptor. When CCR5 is unavailable, CCR5-tropic HIV cannot infect a CD4 cell. The CCR5-tropic variant of the virus is common in early HIV infection, whereas viruses adapted to use the CXCR4 receptor gradually become dominant in some patients as HIV infection progresses. IIs block the catalytic activity of HIV-1 integrase, an enzyme required for viral replication. Integrase inhibition prevents the covalent insertion of unintegrated, linear HIV-1 DNA into the host cell genome, preventing the formation of HIV-1 provirus. Raltegravir is the only II currently in clinical use, although others including elvitegravir are in development. Figure 1.2 (Spach, 2007), shows the current antiretroviral drug targets at the key stages in the HIV Lifecycle.

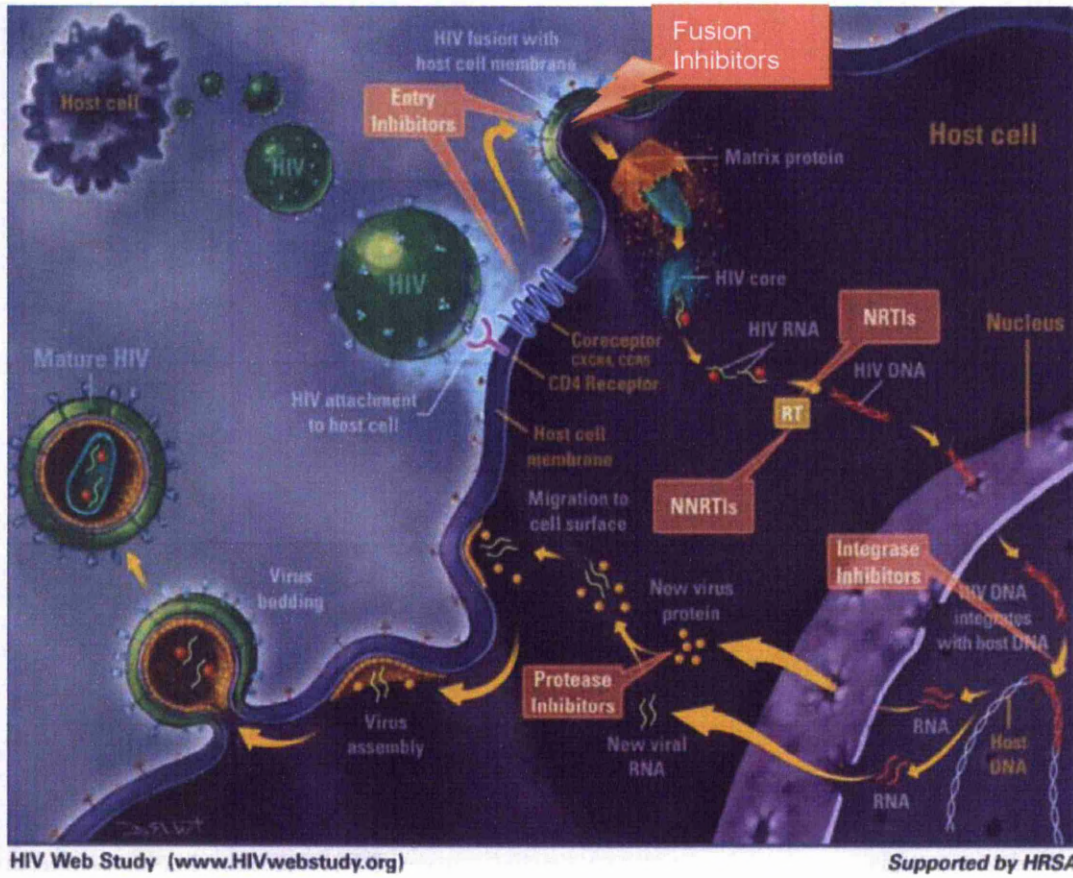


Figure 1.2: HIV life cycle and current drug targets by class (NRTIs - nucleoside reverse transcriptase inhibitors; NNRTIs – non-nucleoside reverse transcriptase inhibitors) – Adapted from HIV Web Study- www.HIVwebstudy.org.

1.3 HIV/HAART-Associated Dyslipidaemic Lipodystrophy (HADL)

The direct toxicity attributed to long-term HAART together with the complex interactions with HIV infection combined with possible other factors, (e.g. genetic predisposition, environmental) influence the risk of developing HIV/HAART-associated dyslipidaemic lipodystrophy (HADL). Successful treatment regimens have greatly improved morbidity and mortality outcomes for HIV+ individuals but the onset of HADL greatly increases the risk of developing cardiovascular disease and associated metabolic complications (Kim *et al.*, 2006b; Mallon, 2007).

HIV infection has been shown to cause changes in lipid metabolism, including decreases in total, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol (Falutz, 2007; Mallon, 2007). Treatment with ARVs (including PIs) has resulted in elevations in total and LDL cholesterol levels whilst HDL cholesterol levels remained low (Mallon, 2007). HIV infection together with the impact of HAART, co-infection with hepatitis C virus (HCV), which is not uncommon in HIV+ cohorts (Buxton *et al.*; Rockstroh *et al.*, 2004), increases the risk of fat redistribution abnormalities and the other metabolic complications associated with HAART (Duong *et al.*, 2001). This together with a genetic predisposition to dyslipidaemias create a complex multi-factorial mechanism leading to an array of metabolic complications (Capeau *et al.*, 2006; El-Sadr *et al.*, 2005; Falutz, 2007; Lagathu *et al.*, 2005). Differences in the pharmacokinetics of many ARVs including ATV have been reported between HIV+ individuals and healthy volunteers (Dickinson *et al.*, 2008). Together these factors have contributed to the identification of wide ranging mechanisms involved in the deleterious metabolic side effects (Behrens *et al.*, 2000; Carr, 2000; El-Sadr *et al.*, 2005; Falutz, 2007; Hruz, 2006; Lee *et al.*, 2005; Mallon, 2007). Single nucleotide polymorphisms (SNPs) in drug disposition genes, transporters and genes involved in lipid homeostasis may play a key role in varied metabolic side-effect profiles seen in HIV+ individuals on HAART (Mallon, 2006; Telenti *et al.*, 2008).

Dyslipidaemia is common in HIV+ individuals on long-term HAART. It is characterised by elevated total and LDL cholesterol, significantly elevated triglycerides together with a decrease in HDL cholesterol, resulting in a profile with

atherogenic potential (Falutz, 2007; Mallon, 2007). The three major classes of ARVs: PIs, NRTIs and NNRTIs, have been implicated in dyslipidaemia, although PIs are most widely implicated (Table 1.1) (Behrens *et al.*, 2000; Carr, 2000; Carr *et al.*, 1998; Falutz, 2007; Mallon, 2006). Insulin resistance is also commonly associated with PIs and NRTIs (Falutz, 2007; Lagathu *et al.*, 2005). PIs have been shown to impact several molecular pathways key to lipid metabolism including intra-nuclear transcription factors and the nuclear proteasome (Lagathu *et al.*, 2005; Mallon, 2007).

Table 1.1: Major classes of approved antiretroviral drugs and associated metabolic complications

Class		Generic Name	Metabolic Complications
Reverse transcriptase (RT) inhibitors	NRTI – (Nucleoside & Nucleotide analogues)	Abacavir	
		Didanosine (ddI)	
		Emtricitabine (FTC)	
		Lamivudine (3TC)	Elevated triglyceride & cholesterol levels
		Stavudine (d4T)	Fat redistribution syndrome, increased insulin resistance, decreased adipocyte differentiation
		Tenofovir (TDF)	
		Zalcitabine (ddC)	
		Zidovudine (AZT)	Increased insulin resistance, decreased adipocyte differentiation
	NNRTI – (Non-Nucleosides)	Delavirdine	Elevated HDL cholesterol levels
		Efavirenz	Elevated triglyceride & cholesterol levels
		Nevirapine	Elevated HDL cholesterol levels
Protease Inhibitors	PIs	Tipranavir	Hyperglycaemia, hyperlipidaemia, fat redistribution, increased insulin resistance (amprenavir)
		Amprenavir	
		Darunavir	
		Fosamprenavir	
		Atazanavir	Lower hyperlipidaemia than other PIs
		Indinavir	Hyperglycaemia, elevated triglyceride levels, increased insulin resistance, hyperlipidaemia, fat redistribution
		Lopinavir/ritonavir	
		Nelfinavir	
		Ritonavir	
		Saquinavir	Hyperglycaemia, hyperlipidaemia, fat redistribution & lipid abnormalities
Entry (EI) & Fusion (FI) inhibitors	EIs & FIs	Enfuvirtide (T20)	Weight loss
		Maraviroc	
Integrase inhibitors	IIs	Raltegravir	

1.4 Adenosine Tri-Phosphate (ATP) Binding Cassette (ABC) Family Transporters

The adenosine tri-phosphate (ATP) binding cassette (ABC) transporters are members of a superfamily of transmembrane proteins which transport a wide variety of endogenous substrates and xenobiotics across extracellular and intracellular membranes. ABC transporters utilise the energy from ATP hydrolysis to facilitate

this transport. ABC transporters play a crucial role in multidrug resistance via the efflux of therapeutic drugs from cells (Ho *et al.*, 2005; Tirona *et al.*, 2005). The main examples of this mechanism covered in this thesis are ABCB1 (P-glycoprotein, P-gp, multidrug resistance protein 1), ABCC1 and ABCC2 (the multidrug resistance associated proteins 1 and 2, respectively) which extrude drugs from human cells, and have been implicated in modulating the levels of anti-retroviral drugs in cells and tissues (Telenti *et al.*, 2002; Telenti *et al.*, 2008). This drug transport system is thought to have evolved as a protective mechanism in which multiple pathways exist for the elimination of potentially harmful xenobiotics. The synergistic or additive functions of these multiple transport systems may need to be considered when examining a particular drug's disposition profile (Ho *et al.*, 2005). Multidrug resistance transporters may contribute to reduced intracellular drug concentrations at sanctuary sites (Pan *et al.*, 2007).

The membrane efflux transporter ABCB1 extrudes diverse hydrophobic peptides and drugs (organic cationic and neutral compounds) from human cells in many key sites for drug metabolism and elimination including the small intestine, liver and kidney (Telenti *et al.*, 2008; Urquhart *et al.*, 2007). ABCB1 has a critical role in drug absorption, biliary excretion, renal secretion and central nervous system (CNS) entry of a wide range of hydrophobic xenobiotics. ABCB1is expressed in tissues which provide barriers to systemic drug exposure and exhibit broad substrate specificity, there is a significant substrate overlap with CYP3A4. Significant inter-individual variability of ABCB1 expression in the intestine and other organs exists (Urquhart *et al.*, 2007). HIV-1 PIs and NNRTIs are known substrates of ABCB1 (Ho *et al.*, 2005;

Telenti *et al.*, 2008). ABCB1 expression is also regulated by the activation of PXR and CAR as previously stated (Kast *et al.*, 2002; Pascussi *et al.*, 2008; Urquhart *et al.*, 2007).

ABCB1and ABCC1 are mainly located in the plasma membrane of the epithelium and endothelium of the liver, intestine, blood-brain barrier (BBB) and the testes (Ho *et al.*, 2005; Pan *et al.*, 2007; Urquhart *et al.*, 2007). Current research seeks to address drug resistance through a variety of mechanisms including the identification of polymorphisms in drug transporters which account for inter-individual variations in response (Cressey *et al.*, 2007; Owen *et al.*, 2006; Rodriguez-Novoa *et al.*, 2006; Telenti *et al.*, 2008).

ABCC2 is a drug efflux transporter which is expressed in the intestine, liver and kidney. Its primary function is the excretion of organic anions from Phase II drug metabolism reactions, e.g. glutathione, glucuronide and sulphate conjugates, from hepatocytes into bile. ABCC2 is also responsible for the transport of endogenous substrates including oestrogens, bilirubin and bile salts (Urquhart *et al.*, 2007). As discussed in detail in sections 1.6.2 and 1.6.3, ABCC2 expression is regulated by the NRs PXR, FXR and CAR. Increased ABCC2 expression has been evidenced following induction of the NRs by the paradigm inducers rifampicin (PXR), chenodeoxycholic acid (FXR) and phenobarbital (CAR) (Urquhart *et al.*, 2007).

The induction of ABCC3 (multidrug resistance associated protein 3) and ABCC4 (multidrug resistance associated protein 4) by NRs has been discussed in detail in section 1.6.1. ABCC3 is a drug efflux transporter expressed on the basolateral

membrane of cells in the liver, kidney and small intestine (Ho *et al.*, 2005; Urquhart *et al.*, 2007). VDR, PXR and CAR are known to induce the expression of ABCC3 whilst SHP (NR0B2) is known to down-regulate its expression (Makishima *et al.*, 2002; Pascussi *et al.*, 2008; Tirona *et al.*, 2005). ABCC4 is a drug efflux transporter in the liver which transports conjugated steroids and bile acids from the hepatocytes into the blood. It has been speculated that ABCC4 compensates for disrupted canalicular bile acid efflux during cholestasis by facilitating transport into the blood for renal excretion (Urquhart *et al.*, 2007). Two polymorphisms in ABCC4 may have clinical relevance in respect of HAART. The NRTIs lamivudine and zidovudine have been associated with elevated intracellular levels linked to T4131G and G3724A respectively (Rodriguez-Novoa *et al.*, 2006). CAR has been shown to induce the expression of MRP4 (Tirona *et al.*, 2005).

1.5 Cytochrome P450 (CYP) Disposition Genes

The gene superfamily of haem proteins, the cytochrome P450 (CYP450, CYP) encodes the main enzymatic system for metabolism of lipophilic substrates of diverse structures and may be subdivided into those which metabolise purely endogenous chemicals and those involved in xenobiotic metabolism. CYPs are important in the oxidative, peroxidative and reductive metabolism of numerous endogenous compounds including steroids, bile acids, fatty acids, prostaglandins, leukotrienes, biogenic amines, and retinoids. Phase I drug metabolism reactions, which involve oxidation, reduction or hydrolysis of the drug, are primarily undertaken by CYPs (Ho *et al.*, 2005).

CYP1A1, CYP1A2 and CYP1B1 are involved in drug and steroid (especially oestrogen) metabolism. The CYP2 subfamily; including the key members CYP2B6, CYP2B10, CYP2C9, CYP2C19, CYP2D6 and CYP2E1, are also involved in drug and steroid metabolism. The NRs PXR, CAR, VDR and LXRα are associated with the regulation of CYP2B6, as discussed in Section1.6. VDR and CAR are also associated with the regulation of CYP2B10 and CYP2C9 (Handschin *et al.*, 2003). The CYP4 subfamily contains the PPAR regulated CYP4A11 and is involved in arachidonic acid and fatty acids metabolism. The CYP7 and CYP8 subfamilies are regulated by PXR, LXR and FXR and are involved in cholesterol homeostasis and bile acid metabolism (Handschin *et al.*, 2003; Tirona *et al.*, 2005).

Human CYP3A4 is the most abundantly expressed hepatic drug metabolising CYP (Martinez-Jimenez *et al.*, 2007) and accounts for the metabolism of more than 50% of clinically used drugs (Lehmann *et al.*, 1998; Luo *et al.*, 2002; Plant, 2007). It is also expressed in the small intestine, where it contributes to the first pass metabolism of many drugs (Luo *et al.*, 2002; Paine *et al.*, 1996). Hepatic and intestinal expression can be induced by a wide variety of therapeutic drugs leading to the accelerated metabolism of the drugs and concomitantly administered therapies leading to altered therapeutic effects and drug-drug interactions (Luo *et al.*, 2002). Inclusion of the PI ritonavir in HIV combination therapies has been shown to significantly improve circulating concentrations of co-administered drugs. Improvement in drug serum concentrations have been attributed to a reduction in overall drug metabolism due to direct inhibition of CYP3A4 by RTV. The NRs PXR,

CAR, VDR, LXRα, GR and HNF4α are all known regulators of CYP3A4 expression.

Human CYP3A subfamily members comprise CYP3A4, CYP3A5, CYP3A7 and CYP3A43. Despite the similarity of DNA sequences the expression pattern of these are quite different. CYP3A4 is predominantly expressed in the adult liver and intestine. CYP3A5 is polymorphically expressed in a variety of tissues, especially the adult liver, kidney and lung. CYP3A7 is expressed in human foetal liver and CYP3A43 is highly expressed in the adult prostate (Luo *et al.*, 2004; Matsumura *et al.*, 2004). There is wide inter-individual variation in the hepatic expression and activity levels of CYP3A4 which has consequences for the efficacy and toxicity of many drugs (Luo *et al.*, 2004; Matsumura *et al.*, 2004).

1.6 Nuclear Receptors

Nuclear receptors (NRs) are the largest known family of transcription factors which function as regulators of tissue gene expression. There are 49 known members of the nuclear receptor superfamily and each member shares key structural features. NRs are divided into 6 NR sub-families based on conserved structure and function (Owen *et al.*, 2000), Figure 1.3 below outlines the phylogenetic tree and evolutionary relationship of the NRs (Benoit *et al.*, 2006). NRs are cellular proteins which are responsible for sensing hormones and xenobiotics. NRs work in concert with other proteins to regulate the expression of specific genes, thereby regulating metabolism, development and homeostasis (Urquhart *et al.*, 2007).

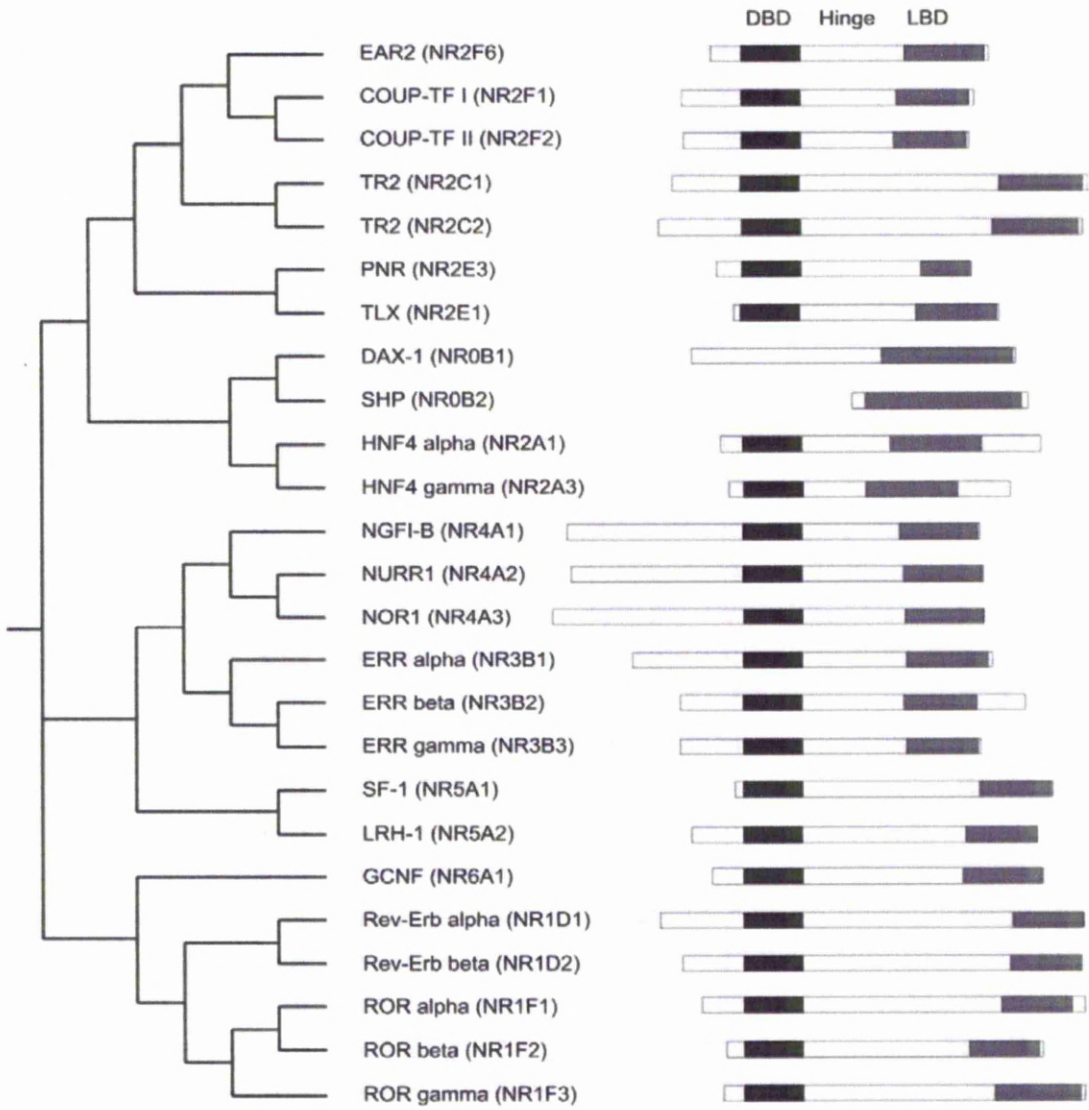


Figure 1.3: A phylogenetic tree and schematic structure of structurally diverse nuclear receptors. Reproduced with permission from Vincent Laudet (Vincent.Laudet@ens-lyon.fr) From Benoit *et al.*, 2006.

NRs have evolved with structural features which are common to the entire NR superfamily (see section 1.6.1 below) whilst ligands for the metabolic NRs are present in high concentrations unlike those for the classical NRs such as the oestrogen receptor (ER), (Francis *et al.*, 2003; Gronemeyer *et al.*, 2004b). Figure 1.4 below illustrates the differences in binding affinity between the metabolic NRs and the oestrogen receptor-α (ERα).

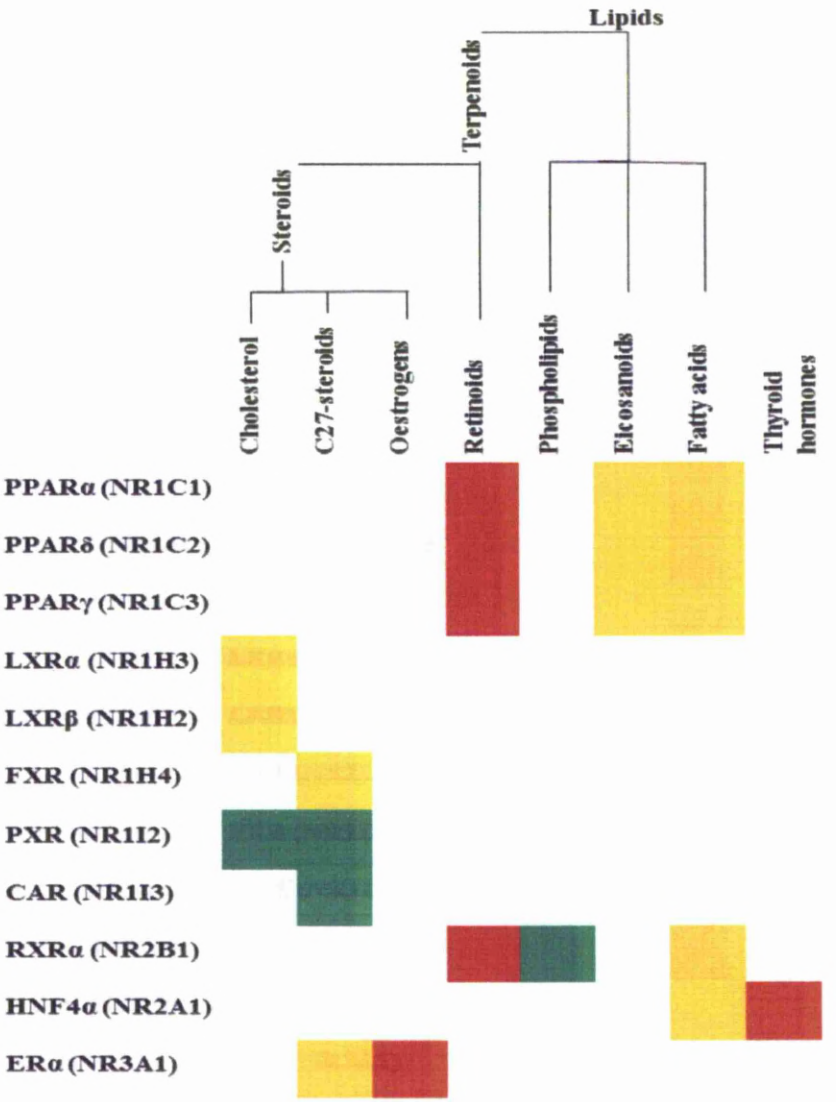


Figure 1.4: The differences in ligand binding affinity between the metabolic nuclear receptors and the oestrogen receptor-α (ERα), Red = nanomolar (nM) binding; Yellow = micromolar (μM) binding; and Green = Binding with variable/unknown affinity.

Over the last twenty years, important new insights have been made in relation to the ligand activated NR mechanisms which regulate the expression of drug metabolising enzymes and transporters. Convincing evidence exists which demonstrates that an array of NRs including: the pregnane X receptor (PXR), constitutive androstane receptor (CAR), farnesoid X activated receptor (FXR), liver X activated receptor

(LXR), vitamin D receptor (VDR), hepatic nuclear factor 4α (HNF4α) and the aryl hydrocarbon receptor (AhR) control the expression of many clinically important drug metabolising enzymes and drug transporters including: the cytochrome P450 (CYP) enzymes: CYP3A4; CYP2B6; CYP2C9; and CYP2C19; Adenosine tri-phosphate binding cassette-B1 (ABCB1); ABCC2; and ABCC4 (Kast *et al.*, 2002; Lehmann *et al.*, 1998; Pascussi *et al.*, 2008; Thummel *et al.*, 2001). The influence of NRs on the regulation of drug disposition genes has greatly influenced the drug development process (Tirona *et al.*, 2005; Urquhart *et al.*, 2007). NRs have evolved to bind distinct ligands and regulate physiological processes, many of these functions have been highly conserved through evolution (Owen *et al.*, 2000).

1.6.1 Nuclear Receptor Structure

NRs comprise three major protein domains: an amino terminal A/B domain or hypervariable region which contains the activation function 1 (AF-1) domain; a centrally located deoxyribonucleic acid (DNA) binding domain (DBD) or C region; and a carboxyl terminus ligand binding domain (LBD) or E/F region (the F region is not found in all NRs (Zhang *et al.*, 2004)), which contains the activation function 2 (AF-2) domain; (Figure 1.5, below) and a hinge region.

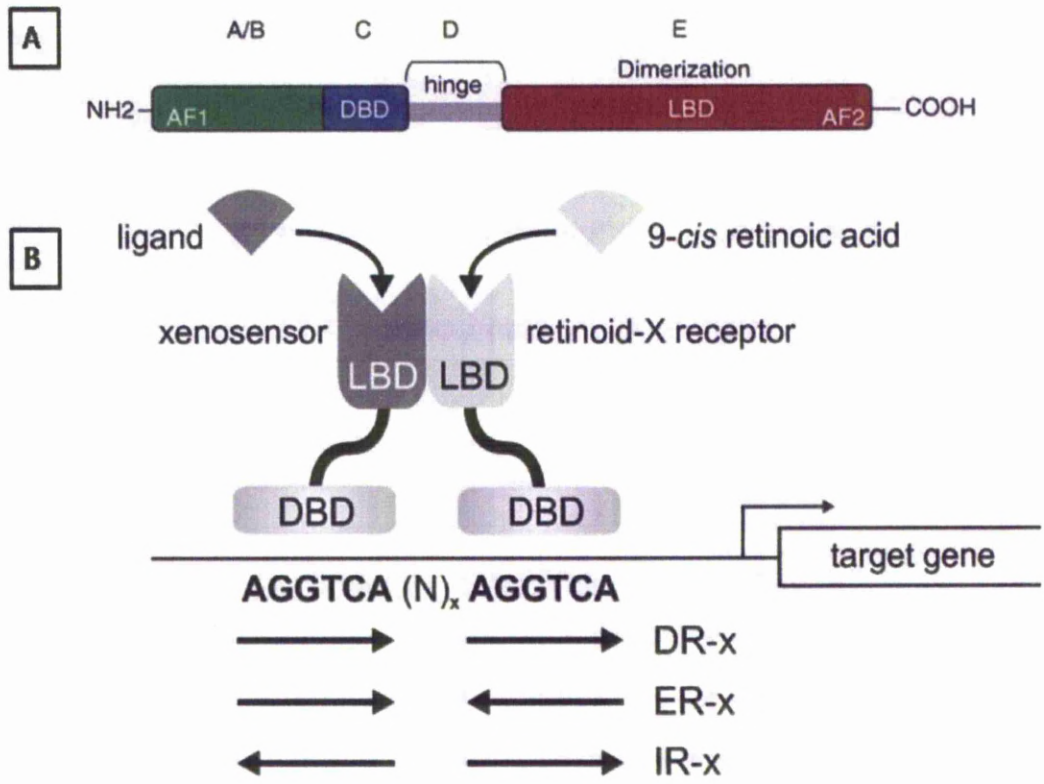


Figure 1.5: Structure and DNA binding of nuclear receptors. **A**: – The molecular structure of a nuclear receptor : The A/B N-terminal with AF-1 region; the C region DBD; the D region - flexible hinge domain; and the E region LBD with AF-2 domain, (the F region is not shown as it is common to all NRs). **B**: - DNA binding of nuclear receptors. The xenobiotic-sensing nuclear receptors bind as heterodimers with RXR to repeats of AG(G/T)TCA with variable spacing. The hexamers can be arranged as direct repeats (DR), everted repeats (ER) or inverted repeats (IR). Comparison of the amino acid sequences of the xenobiotic sensing NRs (PXR and CAR) and closely related NRs VDR, the bile acid sensor FXR, and the cholesterol sensors LXRα and LXRβ reveals a high level of similarity. Adapted from Handschin and Meyer, 2003 and Fiorucci *et al.*, 2007.

These protein domains can be categorised based on function and amino acid position along the polypeptide chain. NR functions are directed by specific activation domains termed AF-1 and AF-2 (Gronemeyer *et al.*, 2004a; Handschin *et al.*, 2003; Rosenfeld *et al.*, 2001; Urquhart *et al.*, 2007). A unique property of NRs which differentiates them from other classes of receptors is their ability to directly interact with and control the expression of genomic DNA (Giguere, 1999). Consequently nuclear receptors play key roles in development and homeostasis of organisms. NRs

may be classified either according to mechanism or homology (Giguere, 1999; Gronemeyer *et al.*, 2004a).

Activation of the acidic AF-1 transactivation domain is ligand-independent or constitutively functional via the recognition of other transcription factors and co-activators. The AF-1 contains sites targeted for phosphorylation by a variety of signalling pathways. Activation effects due to phosphorylation are receptor-specific and can result in either enhanced or diminished receptor activation. The AF-2 domain is located within the carboxyl terminus LBD. Although AF-1 and AF-2 are capable of regulating transcription alone, full transcriptional activation by AF-2 requires functional interplay between the two regions. This interaction facilitates sustained receptor activation and robust gene transcription through stabilisation of helix 12, which prevents premature dissociation of the ligand, and mutual binding of co-activators (Francis *et al.*, 2003; Gronemeyer *et al.*, 2004a; Urquhart *et al.*, 2007).

The DBD interacts with DNA response elements (RE) on target genes to modulate their transcription. The DBD is GC-rich and contains two zinc finger motifs with a carboxyl-terminal extension containing T and A boxes which are critical for monomeric DNA binding of the RE half-sites. The DBD is the most highly conserved region across the NR superfamily. The first zinc finger contains the proximal (or P-box) region which is responsible for high affinity recognition of the core half site of the RE. The distal (or D-box) region is located within the second zinc finger and is a site which mediates receptor dimerisation (Khan *et al.*, 2003).

The LBD varies greatly between NRs but all share a common structure of twelve α-helices organised around a hydrophobic binding pocket for lipophilic ligand-binding. Residues within the binding pocket confer specificity. Ligand-dependent transactivation requires the AF-2 domain which is located at the extreme terminus of the LBD. The LBD binds an array of small, lipophilic endogenous ligands including hormones, fatty acids, oxysterols, bile acids and lipids, together with exogenous ligands including drugs and other chemicals (xenobiotics) (Urquhart *et al.*, 2007). The LBD is multifunctional with sequences critical for nuclear localisation, receptor dimerisation and the binding of heat shock proteins (HSPs), co-regulators and other transcription factors. Binding of the ligand triggers the rearrangement of the LBD helices which initiates the release of the HSPs and co-repressors (Francis *et al.*, 2003; Giguere, 1999; Handschin *et al.*, 2003).

The hinge or D region is located between the DBD and LBD. The hinge region is variable in both length and sequence between different NRs. It contains binding sequences for co-repressors which are essential for transcriptional silencing. It allows for conformational changes in the protein structure once the ligand is bound, and provides flexibility to the receptor domains for proper orientation for both RE binding and dimer formation (Khan *et al.*, 2003; Rosenfeld *et al.*, 2001).

The REs are comprised of two hexameric nucleotide sequences known as half-sites. The sequence, arrangement and spacing of these half-sites determine the responsiveness of a gene to the NRs. Steroid NRs typically bind as homodimers preferentially recognising half-sites with the sequence AGAACA. NRs which form a

heterodimer with RXR recognise the half site sequence AGGTCA with variable spacing between the two half-sites (Figure 1.5). Receptor specificity is conveyed by the nucleotide spacing of the half-sites together with the arrangement as direct (DR) (AGGTCA-Nx-AGGTCA), inverted (IR) (AGGTCA-Nx-ACTGGA) or everted repeats (ER) (ACTGGA-Nx-AGGTCA), (N is any nucleotide and x is any number of residues between 0 and 10) (Khan *et al.*, 2003). Table 1.2 below illustrates the response elements recognised by the key metabolic NRs and identifies the mechanisms involved in NR functional redundancy and crosstalk.

Table 1.2: The binding arrangements and response elements recognised by the key metabolic NRs together with a summary of the mechanisms involved in NR Cross-talk.

	Monomer, Homodimer or Heterodimer	Half site recognition sequence	Response Element	Receptor Crosstalk	Mechanisms
PPARα (NR1C1)	Heterodimer	AGGTCA	DR-1		
PPARγ (NR1C3)	Heterodimer	AGGTCA	DR-1	PPARγ/PXR	Induction of PPARγ by activated PXR
LXRα (NR1H3)	Heterodimer	AGGTCA	DR-4	CAR-PXR/LXR	Inhibition of CAR-PXR transcriptional activity by activated LXR
LXRβ (NR1H2)	Heterodimer	AGGTCA	DR-4		
FXR (NR1H4)	Heterodimer	AGGTCA	DR-4	CAR-PXR/FXR	FXR and PXR share agonists;
PXR (NR1I2)	Heterodimer	AGGTCA & AGTTCA AG(G/T)CA	DR-3 (distal enhancer region) DR-4 ER-6 (in the proximal promoter region) ER8		FXR transactivates CAR-PXR target genes
CAR (NR1I3)	Monomer or heterodimer	AGGTCA & AGTTCA AGGTCA AG(G/T)CA	DR-3 (distal enhancer region) DR-4 DR-5 ER-6 (in the proximal promoter region)	PXR-CAR/VDR	PXR-CAR/VDR Share response elements.
VDR (NR1I1)	Heterodimer	AGGTCA TGAACT	DR-3 (distal enhancer region) DR-4 ER-6 (in the PXRE ER6 motif in the CYP3A4 proximal promoter or in the VDRRE proximal promoter)		
RXRα (NR2B1)	Homodimer & Heterodimer partner	AGGTCA	DR-1 DR-4		
HNF4α (NR2A1)	Homodimer	AGGTCA	DR-1 DR-2		
ERα (NR3A1)	Homodimer	AGAACA	IR-3		

Activation of the NRs starts in the cytoplasm where the NRs typically reside bound to transcriptional co-repressors. The co-repressors recruit specific histone deacetylase-containing complexes, which facilitate the regulation of DNA transcription (Legube *et al.*, 2003). Drug ligands pass through the cell membrane, by either diffusion or selective transport, and bind to the LBD causing dissociation of the histone deacetylase-containing complex and receptor translocation to the nucleus. Once in the nucleus, the ligand-receptor complex recruits co-activators and forms either a homodimer or in most cases heterodimerises the retinoid X receptor (RXR). The homodimer or heterodimer complex then binds to the DNA response elements (RE) in the regulatory (promoter and enhancer) regions of target genes (Urquhart *et al.*, 2007). NR activation can also be influenced by other mechanisms including phosphorylation by cellular kinases in cell signalling pathways and protein-protein interactions through contacts with other transcription factors (Spiegelman *et al.*, 2004).

Inactivated NRs generally form a complex with co-repressors including RIP140, nuclear receptor co-repressor 1 (NCoR1) and silencing mediator for retinoid and thyroid hormone receptor (SMRT, also known as NCoR2) which inhibit transcriptional activity, often through histone deacetylase recruitment (Francis *et al.*, 2003; Pascussi *et al.*, 2008). Receptor activation by ligand binding or phosphorylation induces a conformational change resulting in co-repressor dissociation and co-activator recruitment (Figure 1.6). Co-activators associated with NR activation include the p160 family (SRC-1 (steroid receptor co-activator 1)), TIF2, GRIP-1, ACTR), p/CIP, CBP/p300, PGC-1, PRIP, PGC-2 and ARA70

(Francis *et al.*, 2003; Handschin *et al.*, 2003; Pascussi *et al.*, 2008). The specific co-regulators recruited by NRs depend on the ligand induced conformational changes, and the promoter and cellular context (Francis *et al.*, 2003; McKenna *et al.*, 1999).

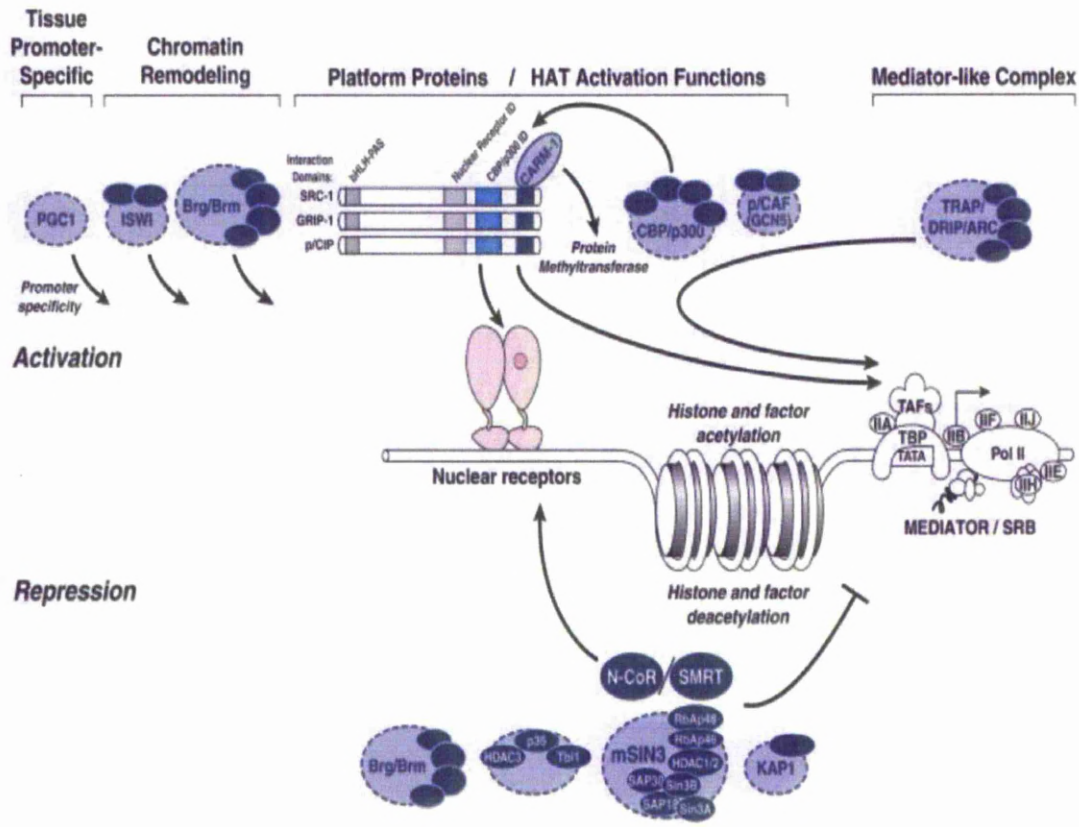


Figure 1.6: Activation and repression of nuclear receptors regulated by ligand - control of recruitment of either co-activators or co-repressors. From Rosenfeld, M. G. *et al.* J. Biol. Chem., 2001.

Peroxisome proliferator-activated receptor gamma co-activator-1 alpha (PGC-1α) has recently been shown to play a critical role in xenobiotic metabolism (Pascussi *et al.*, 2008). PGC-1α has no intrinsic histone acetyl transferase activity, which prevents it from regulating DNA transcription in its own right (Finck *et al.*, 2006; Legube *et al.*, 2003). When PGC-1α is docked to transcription factors or NRs, however, it provides a platform for recruiting co-activators with histone acetyl transferase

activity (Finck *et al.*, 2006; Legube *et al.*, 2003). PGC-1α plays a key role in cellular energy metabolism through co-ordination of transcriptional programs of mitochondrial biogenesis, adaptive thermogenesis and fatty acid β-oxidation. PGC-1α is involved in the co-activation of key transcription factors of hepatic gluconeogenesis (Glass, 2006; Pascussi *et al.*, 2008). Hepatic PGC-1α expression is induced by fasting. PGC-1 isoforms affect the activity of multiple NRs including PPARγ, RXR, GR, FXR, LXR, HNF4α, PXR and CAR (Pascussi *et al.*, 2008). Specific NR co-regulator complexes fine tune the biological response to ligand receptor interaction and underpin the variability of gene responses to different ligands and metabolic environments (Francis *et al.*, 2003; Gronemeyer *et al.*, 2004a; Handschin *et al.*, 2003; Tirona *et al.*, 2005).

Constitutive and inductive expression of drug disposition genes is under co-ordinate transcriptional control by NRs. Functional REs for NRs within a gene may bind different NRs, e.g. REs in CYP2B, CYP2C and CYP3A can be transactivated by PXR, CAR and VDR providing a level of redundancy to environmental xenobiotic exposure (Ho *et al.*, 2005; Tirona *et al.*, 2005). PXR and CAR mediated induction of CYP2B, CYP2C and CYP3A genes is regulated by GR signalling (Handschin *et al.*, 2003). NR regulation complexity is highlighted in the modulation of CYP3A4 expression (Handschin *et al.*, 2003; Ho *et al.*, 2005; Smirlis *et al.*, 2001; Thummel *et al.*, 2001; Tirona *et al.*, 2003). The effect of PXR and CAR induction on CYP3A4 expression is critically dependent on HNF4α acting on the distal enhancer (Pascussi *et al.*, 2008; Tirona *et al.*, 2003; Urquhart *et al.*, 2007). The combined actions of the

transcription factors C/EBPα and AP-1 together with HNF4α influence the CYP3A4 expression (Tirona *et al.*, 2003).

NR mediated induction can be mutually repressed by other NRs and transcription factors. PPARγ and FXR can repress CAR mediated gene induction by co-activator competition. LXRα can directly compete with CAR and PXR on CYP3A and CYP2B NR REs to suppress transcription. An emerging theme in regulation of gene expression is the interconnected regulation of NR expression by other NRs. HNF4α, and GR are particularly important in regulating the expression of other ligand-activated transcription factors such as PXR, CAR and FXR. PXR also regulates the expression of CAR and itself (Francis *et al.*, 2003; Gronemeyer *et al.*, 2004a; Pascussi *et al.*, 2008; Tirona *et al.*, 2005). The complex nature of the metabolic NRs involved in bile acid metabolism and detoxification together with the drug disposition genes and transporters regulated by the activation of the NRs is outlined in Figure 1.7, below.

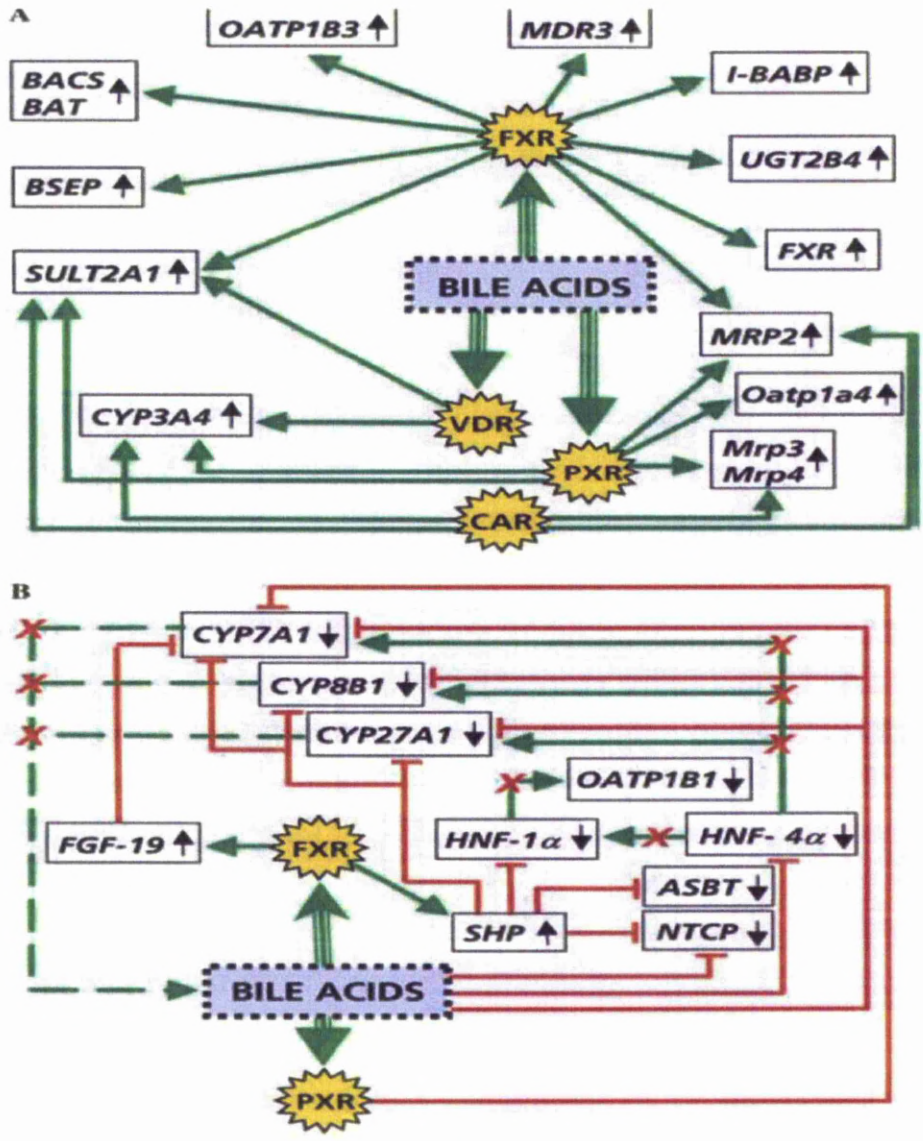


Figure 1.7: Metabolic NRs involved in bile acid metabolism: Green lines = transcriptional activation, red lines = transcriptional suppression (A) Genes activated by bile acid dependent mechanisms. (B) Genes suppressed by bile acid-mediated mechanisms. Discontinuous green lines = bile acid synthesis. Red crosses over green lines = bile acid-mediated suppression of otherwise stimulatory pathways. From Eloranta *et al.*, Arch Biochem Biophys, 2005.

The PIs ritonavir (RTV) and atazanavir (ATV) have been shown to be substrates for/activators of the NRs including PXR and CAR (Dussault *et al.*, 2001; Healan-Greenberg *et al.*, 2008; Lehmann *et al.*, 1998). PIs have also been shown to impact several molecular pathways key to lipid metabolism including intra-nuclear

transcription factors and the nuclear proteasome (Lagathu *et al.*, 2005; Mallon, 2007). Previous studies have focused on the roles of PXR and CAR in the metabolism of RTV and ATV with limited investigation of the role of the metabolic NRs controlling lipid and bile homeostasis in response to PI metabolism. FXR and LXRα are master regulators of cholesterol homeostasis, and modulate the transcription of many key genes involved in drug and lipid metabolism including the sterol regulatory element binding protein-1c (SREBP-1c), the most abundant sterol regulatory binding protein (SREB) isoform in adult human liver and a key player in lipogenesis (Hui, 2003; Liang *et al.*, 2001; Osborne, 2000).

RTV is no longer used as a PI in highly active antiretroviral therapy (HAART) but it is used at low dose to boost the activity of other PIs through its inhibition of the cytochrome P450 (CYP) enzyme CYP3A4. RTV has no intrinsic antiviral activity at low dose but it has been widely implicated with hypertriglyceridaemia (Kim *et al.*, 2006b; Mallon, 2007; Purnell *et al.*, 2000). The pathogenesis of the RTV-induced effects has been linked to the effect of RTV on hepatic and adipocyte SREBs including SREBP-1c (Hui, 2003; Liang *et al.*, 2001; Osborne, 2000). RTV has also been associated with enhancing the formation of very low density lipoproteins (VLDL). A study in the human adipocyte cell line, Simpson Golabi Behmel Syndrome (SGBS), has shown that ATV is associated with fewer signs of the deleterious metabolic side effects seen in lipodystrophy (Kim *et al.*, 2006b). Clinical trials have also shown that ATV has a better lipid profile than other PIs including RTV (Flammer *et al.*, 2009; Fuster *et al.*, 2005). Co-infection with HIV and hepatitis C virus (HCV) increases the risk of fat redistribution abnormalities and the other

metabolic complications associated with HAART (Duong *et al.*, 2001). Differences in the pharmacokinetics of many ARVs including ATV have been reported between HIV+ individuals and healthy volunteers (Dickinson *et al.*, 2008). Current research into metabolic disorders has included investigations into the role of NRs including PXR, PPARα, PPARγ, FXR, LXR and VDR in these disorders (Cha *et al.*, 2007; Edwards *et al.*, 2002; Fiorucci *et al.*, 2007; Francis *et al.*, 2003).

1.6.2 Pregnane X Receptor (PXR) and Constitutive Androstane Receptor (CAR)

The pregnane X receptor (PXR, NR1I2) is expressed in the liver (predominantly), small intestine, colon, kidney, lung, uterus, ovary, breast, placenta and lymphocytes (Chang *et al.*, 2005; Francis *et al.*, 2003; Tirona *et al.*, 2005). It is activated by a broad range of structurally diverse xenobiotics and endogenous ligands. PXR plays a key role in the detoxification and clearance of xenobiotics; PXR REs have been identified in CYPs, drug efflux transporters and phase II drug metabolising enzymes (Goodwin *et al.*, 2002; Maglich *et al.*, 2002; Waxman, 1999).

PXR is involved in the up-regulation of CYP1A1, CYP1A2, CYP2A4, CYP2A6, CYP2B6, CYP2B10, CYP2C8, CYP2C9, CYP2C19, CYP3A4, CYP3A7, CYP3A11 and CYP24A1 and the down regulation of CYP7A1 (Handschin *et al.*, 2003; Luo *et al.*, 2002; Maglich *et al.*, 2002; Plant, 2007; Staudinger *et al.*, 2001; Tirona *et al.*, 2005). PXR target genes also include the UDP-glucoronosyltransferases (UGTs) 1A1, 1A3, 1A4 and 1A6, glutathione S-transferase A2 (GSTA2), dehydroepiandrosterone sulphotransferase (SULT2A1) and organic anion transporting polypeptides (Chang *et al.*, 2005; Maglich *et al.*, 2002; Rosenfeld *et al.*, 2001). PXR initiates up-regulation by the binding of the PXR-RXR heterodimer to a

xenobiotic responsive enhancer module (XREM) on its target CYP gene. PXR initiates increased expression of the drug transporters ABCB1 and ABCC2 (Tirona *et al.*, 2005). Recent studies suggest that many clinically significant drug-drug interactions involving CYP3A induction are largely a consequence of drug–mediated activation of PXR (Luo *et al.*, 2004; Matic *et al.*, 2007). PXR RE sequences identified in the promoter region of the CYP3A4 gene have indelibly linked its transcriptional regulation to the activation of PXR (Lehmann *et al.*, 1998). Activators of PXR are mainly comprised of substrates and inhibitors of CYP3A4 which provides a mechanistic loop for effective drug clearance (Dussault *et al.*, 2001).

PXR is associated with the co-repressors: small heterodimer partner (SHP), RIP140, NCoR and SMRT which inhibit transcriptional activity (Francis *et al.*, 2003; Pascussi *et al.*, 2008). Co-activators associated with PXR activation include SRC-1, GRIP, PGC-1α and PBP (Francis *et al.*, 2003; Handschin *et al.*, 2003; Pascussi *et al.*, 2008). Increased gene transcription by PXR is initiated by ligand binding to the receptor followed by cytoplasmic nuclear translocation. HIV PIs are known activators of PXR and have been shown to increase expression of CYP3A4. Indinavir and nelfinavir are not thought to bind PXR (Dussault *et al.*, 2001).

PXR activation involves the ligand binding to the receptor, heterodimerisation with RXR, binding of the RXR heterodimer to REs of target genes, release of co-repressor proteins, and recruitment of co-activators and transcription factors. PXR-RXR heterodimers bind to hexameric sequence pairs (5'-AG(G/T)TCA-3') arranged as a

DR3 in the distal enhancer region, DR4, ER6 in the proximal promoter region, or ER8.

In addition to its role as a xenobiotic sensor, PXR has been linked to the regulation of lipid homeostasis. Activated PXR inhibits forkhead box A2 (FoxA2) transcriptional activity leading to a decrease in fatty acid metabolism (Pascussi *et al.*, 2008). It has a regulatory role in lipid and cholesterol pathways via activation by endogenous sterol metabolites. PXR is activated in the presence of high levels of bile acids, especially lithocholic acid (LCA), and oxysterols in the liver (Staudinger *et al.*, 2001). PXR activation results in down-regulation of CYP7A1, the rate-limiting enzyme involved in the conversion of cholesterol to bile acids, a role also performed by FXR. Recent data suggests that FXR and PXR may co-operate to promote robust liver detoxification in the presence of high levels of bile acids to facilitate LCA clearance (Staudinger *et al.*, 2001). PXR also plays an important role in lipogenesis through its activation of the fatty acid transporter Cluster of Differentiation 36 (CD36) and accessory lipogenic enzymes including Stearoyl-CoA desaturase 1 (SCD-1) and long chain free fatty acid elongase (Zhou *et al.*, 2006).

PXR is implicated in the regulation of gluconeogenic gene transcription (Kodama *et al.*, 2004; Pascussi *et al.*, 2008). Regulation of gluconeogenic enzymes by PXR occurs through crosstalk with the forkhead transcription factor (FOXO1). FOXO1 is an insulin responsive transcription factor which increases gluconeogenic gene transcription including phosphoenolpyruvate carboxykinase 1 (PEPCK1) and glucose-6-phosphatase (G6Pase). FOXO1 activity is inhibited through insulin

signalling upon feeding. PXR activation inhibits FOXO1 activity by blocking the binding of FOXO1 to insulin response sequences in the promoters of target genes. Crosstalk regulation at FOXO1 may be needed to control the balance between the cofactors NADPH and NADH. PXR inactivation of gluconeogenesis would increase availability of NADPH for use by drug metabolising enzymes including CYP3A4, for which NADPH is an essential electron donor (Kodama *et al.*, 2004).

Many xenobiotics interact with both PXR and CAR as agonists, inverse agonists or activators. Phenobarbital, oestrogen and the oestrogen precursor, DHEA, are activators of both PXR and CAR. Androstanol is a PXR activator but an inverse agonist of CAR. Bile acids including cholic acid, 12-ketocholic acid and its derivatives are primary ligands of LXR and FXR, activators of PXR and suppressors of CAR. PXR and CAR induce an overlapping series of genes and, as a consequence, primary activation pathways are dependent upon the respective levels of the particular receptor. CITCO (6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl) oxime) has been developed as a specific CAR activator to discriminate between the activation of human PXR or human CAR (Pascussi *et al.*, 2008).

The constitutive androstane receptor (CAR, NR1I3) is expressed in the liver, intestine and lung, and is involved in the up-regulation of the drug metabolising genes CYP1A1, CYP2A4, CYP2A6, CYP2B6, CYP2B10, CYP2C9, CYP2E1, CYP3A4 and CYP3A11. CAR is also implicated in the increased expression of the drug transporters ABCC2, ABCC3 and ABCC4 (Tirona et al., 2005). CAR binds to

hexameric REs (5'-AG(G/T)TCA-3') arranged as a DR3 in the distal enhancer region, DR4, DR5 (5'-AGGTCA-3') (as a heterodimer with RXR or ER6 (5'-AG(G/T)TCA-3') in the proximal promoter region (Francis *et al.*, 2003; Giguere, 1999; Goodwin *et al.*, 2004; Pascussi *et al.*, 2008).

CAR activity is suppressed by its endogenous inverse agonist ligands androstenol and androstanol which act by causing the dissociation of co-activator proteins (Giguere, 1999). CAR activation by phenobarbital reverses the action of the endogenous inverse agonists and suggests that CAR, like PXR and PPARα, participates in a NR mediated CYP induction pathway in response to exogenous xenobiotics and endogenous lipids and steroids (Giguere, 1999). Activated CAR translocates to the nucleus where it initiates the transcription of genes including members of the CYP2B family. CAR plays an important role in regulating bilirubin clearance and bile acid detoxification via crosstalk with PXR, FXR, LXR and SHP (Pascussi *et al.*, 2008).

1.6.3 Farnesoid X Activated Receptor (FXR) and Liver Oxysterol Activated Receptor (LXR)

The farnesoid X activated receptor (FXR, NR1H4) is expressed in the liver, intestine, kidney, adrenal cortex, gall bladder and vascular endothelium (Francis *et al.*, 2003; Gronemeyer *et al.*, 2004a; Handschin *et al.*, 2003; Pascussi *et al.*, 2008). FXR is involved in the down regulation of CYP7A1 and CYP8B1. FXR regulates the increased expression of ABCC2 and ABCB11 (the bile salt export pump, BSEP). FXR shares agonists with PXR leading to increased xenobiotic metabolism by bile

acids. FXR also transactivates PXR and CAR target genes (Pascussi *et al.*, 2008; Tirona *et al.*, 2005).

FXR and the liver X activated receptor alpha (LXRα) play a pivotal role in total body cholesterol homeostasis. FXR, the counterbalance of hepatic LXR action, is activated by bile acid accumulation resulting from the LXR-mediated conversion of cholesterol to bile acids. Bile acids are important for the solubilisation and transport of dietary lipids and are the major products of cholesterol catabolism. High concentrations of bile acids can be toxic to the cell and FXR functions to minimise this by inhibiting further bile acid production. Inhibition of bile acid synthesis is achieved by FXR down regulating CYP7A1, the rate-limiting enzyme in bile acid synthesis, through indirect signalling involving the increased expression of the SHP-1 liver receptor homolog-1 (LRH-1) pathway. SHP-1 represses CYP7A1 expression by binding to promoter sequences and preventing further RE binding of activated LXR (Eloranta *et al.*, 2005).

FXR facilitates the elimination of bile acids through the activation of bile acid inducing enzymes and transport processes including UGT2B4, SULT2A1, BSEP and the intestinal bile acid binding protein (IBABP) (Francis *et al.*, 2003; Giguere, 1999; Pascussi *et al.*, 2008). FXR impacts drug disposition genes via the formation of heterodimers with RXR and has a similar mechanism of action to PXR. FXR-RXR heterodimers bind to hexameric sequence pairs (5'-AGGTCA-3') arranged as a DR4 or an IR1 (Francis *et al.*, 2003; Giguere, 1999; Gronemeyer *et al.*, 2004a; Pascussi *et al.*, 2008).

Recent publications have shown that FXR activation has positive effects on glucose metabolism. Positive effects of FXR agonists on glucose tolerance are brought about through the hepatic activation of FXR, the resulting repression of gluconeogenic genes (PEPCK and G6Pase) and increased hepatic glycogen synthesis. FXR activation improves serum lipid profiles through suppression of hepatic triglyceride synthesis via repression of SREBP-1c and increased expression of lipid metabolism genes including PLTP, LCAT and ApoC-II (Fiorucci *et al.*, 2007). SREBP-1c is a target gene of FXR via a SHP-1 dependent pathway. The consequence of FXR down-regulation of SREBP-1c *in vivo* would be the repression of hepatic triglyceride and fatty acid synthesis (Zhang *et al.*, 2008), although FXR mediated inhibition of fatty acid synthesis may be transient. Prolonged exposure to high levels of bile acids or potent agonists does not appear to have the same beneficial effects on plasma lipids as short term exposure. SHP-1 mediated suppression of SREBP-1c via FXR activation appears to be overcome through the direct activation of fatty acid synthase (FAS) by FXR which is necessary for sufficient fatty acid synthesis under states of cholesterol and bile acid excess to avoid cholesterol toxicity (Fiorucci *et al.*, 2007).

LXRα (NR1H3) is expressed in the liver (predominant), intestine, kidney, adipose tissue, macrophages, ovary, brain, lung and spleen. LXRβ (NR1H2) has ubiquitous tissue and organ expression. LXRα is a regulator of cholesterol homeostasis in the liver and macrophages (Gronemeyer *et al.*, 2004a; Kalaany *et al.*, 2006; Pascussi *et al.*, 2008). It facilitates the processing and storage of cholesterol by up-regulating the cytochrome P450 7A1 enzyme (CYP7A1) and is implicated in the increased expression of ABCA1 (cholesterol efflux regulatory protein, CERP), ABCG1,

ABCG5 and ABCG6 (Eloranta *et al.*, 2005; Pascussi *et al.*, 2008; Tirona *et al.*, 2005). LXRα is also abundant in adipose tissue, where its activation has been shown to increase basal glucose uptake and the incorporation of TGs into lipid droplets (Lehrke et al., 2005). The most potent activating oxysterol ligands of LXRα have been identified as 24(S), 25-epoxycholesterol, 22(R)-hydroxycholesterol and 24(S)-hydroxycholesterol (Edwards *et al.*, 2002). These potent ligands are known to activate both LXRα and LXRβ (Bradley *et al.*, 2005).

The transcriptional activity of LXRα is induced by elevated levels of naturally occurring oxysterols, which increase proportionally to cellular cholesterol content, and 6α-hydroxy bile acids (Eloranta et al., 2005; Francis et al., 2003). LXR responds to high cholesterol levels by increasing fatty acid synthesis to increase the formation of cholesterol esters, rendering the excess cholesterol pool biologically inactive (Giguere, 1999; Pascussi *et al.*, 2008; Urquhart *et al.*, 2007). LXR also facilitates efflux of cholesterol from the periphery, including macrophages, through the increased expression of ABC transporters. Induction of ABC transporters increases the transfer of cholesterol and phospholipids to apolipoprotein A-I (ApoA-I) containing lipoproteins. Activation of LXRα decreases intestinal absorption of cholesterol mediated through the induction of ABC transporters including ABCG5 and ABCG8 (Kalaany *et al.*, 2006; Pascussi *et al.*, 2008).

The effects of LXRα on fatty acid synthesis are mediated through the increased expression of SREBP-1c, a stimulator of lipogenesis. SREBP-1c is a transcription factor which has been previously associated with PI induced lipodystrophy (Bastard

et al., 2002; Telenti *et al.*, 2002). Increased production of liver triglycerides alongside transient increases in plasma levels resulting from the up-regulation of SREBP-1c remains a hurdle in the development of therapeutic LXRα agonists. LXR mediated improvement in glucose tolerance is associated with decreased expression of rate-limiting enzymes of gluconeogenesis including PEPCK and G6Pase, and the induction of genes for glucose uptake and utilisation in the liver (L-GK) and periphery (GLUT4) (Kalaany *et al.*, 2006).

Studies in LXR null mice identified the function of LXR as a sensor of cellular oxysterols and a regulator of the target gene expression of CYP7A1, ABCG1, ABCA1, ApoE, CETP, SREBP-1c, LPL, LXRα self-regulation, FAS, ABCG5, ABCG8, ApoC-I, ApoC-II, ApoC-IV and PLTP (Edwards *et al.*, 2002; Joseph *et al.*, 2003). These LXR target genes all encode proteins which have major roles in controlling cholesterol and fatty acid homeostasis in tissues which include the liver, intestine, macrophages and possibly adipose tissue (Edwards *et al.*, 2002). Studies in murine models have shown that LXR agonists exert desirable antiatherogenic effects in macrophages, but they also increase hepatic and serum triglyceride levels (Bradley *et al.*, 2005; Peet *et al.*, 1998a; Peet *et al.*, 1998b). The hepatic expression of key fatty acid metabolism genes involved in LXRα mediated lipogenesis is reduced in LXRα knockout mice, these include SREBP1-c, FAS and SCD-1. LXRβ knockout mice, however, do not show a similar phenotype (Bradley *et al.*, 2005; Peet *et al.*, 1998b).

LXR-RXR heterodimers bind to DR4 (5'-AGGTCA-3') REs (Francis *et al.*, 2003). LXRα and LXRβ transcriptional activity is induced by naturally occurring oxysterols and 6α-hydroxy bile acids (Eloranta *et al.*, 2005; Francis *et al.*, 2003). LXRs regulate the metabolism of several important lipids, including cholesterol and bile acids. LXR-β is an oxysterol-activated member of the LXR subfamily of the nuclear receptor superfamily of transcription factors. Like its genetic homologue LXRα, LXRβ is an important regulator of cholesterol homeostasis in the liver and macrophages (Bradley *et al.*, 2005; Kalaany *et al.*, 2006; Pascussi *et al.*, 2008). Schultz *et al* hypothesised that LXRα was capable of compensating for the loss of LXRβ in LXRβ double knockout mice as there was no obvious loss of lipid phenotype in these mice in response to cholesterol feeding (Schultz *et al.*, 2000). LXRβ was unable to compensate for the loss of LXRα in LXRα deficient mice in the regulation of SREBP1-c expression in the presence of LXR and RXR agonists and dietary cholesterol, suggesting that LXRα is the more important regulator of this pathway (Millatt *et al.*, 2003).

1.6.4 Retinoid X Receptor (RXR)

The retinoid X receptor (RXR) family of NRs is comprised of three isotypes, α, β and γ. RXRα is the dominant RXR isoform and the preferred heterodimer partner of metabolic NRs including the PPARs, PXR, VDR, LXRs and FXR. Along with other members of the RXR family, 50-80% of retinoids are stored in liver stellate cells. 15-20% of the remaining retinoids are stored in adipose tissue via direct import from the bloodstream through the cellular retinol-binding protein, and via the LPL-mediated uptake of retinyl esters from chylomicrons. RXRβ and RXRγ have a role in regulating the effects of retinoic acid but are outside the scope of this research.

RXRα. (NR2B1) is ubiquitously expressed and is involved in the up-regulation of drug metabolising genes through its role as a heterodimer partner of other NRs (Giguere, 1999; Gronemeyer *et al.*, 2004a; Pascussi *et al.*, 2008; Tirona *et al.*, 2005). RXRα can form permissive heterodimers (can be activated by either the partner ligand or RXRα ligand) or non-permissive heterodimers (can only be activated by the partner ligand). FXR and LXRα form permissive heterodimers with RXRα (Eloranta *et al.*, 2005). RXRα is activated by 9-*cis* retinoic acid, a product of vitamin A (retinol) metabolism which can promote the formation of RXR-RXR homodimers which bind to a DR1 RE (Giguere, 1999).

Phytanic acid, a fatty acid metabolite of chlorophyll found in the human diet, is a specific RXR ligand (Giguere, 1999). RXR affinity for fatty acids negates the possibility that RXR, like its heterodimer partners, including LXRα, FXR and the PPARs, also operates as a sensor of energy metabolism with the potential to drive metabolic responses through the concerted activation of RXR heterodimers (Kassam *et al.*, 2003; Repa *et al.*, 2000). The similarity of effects may be due to the preferential activation of the RXR/PPARγ heterodimer by RXR activators. RXRα selective ligands known as rexinoids have been suggested as potential anti-diabetic agents for the treatment of insulin-independent (Type II) diabetes (Vivat-Hannah *et al.*, 2005). The therapeutic potential for the development of RXRα ligands to treat long term disorders in counterbalanced by the toxic side effects of retinoids, including bone toxicity and hyperlipidaemia, although the latter is more widely associated with the retinoic acid receptors (RARs) (Vivat-Hannah *et al.*, 2005). The RXRα ligand, 9-*cis* retinoic acid, has been shown to be a potent activator of LXR

transcriptional activity (Giguere, 1999). Furthermore, the HIV PIs RTV, saquinavir (SQV) and nelfinavir (NFV) have been shown to block adipogenesis and increase lipolysis *in vitro* in studies in the presence of insulin and agonists for RXR and PPARγ (Lenhard *et al.*, 2000). RXRα plays roles in a variety of processes including embryonic patterning and organogenesis, cell proliferation and differentiation.

1.6.5 Vitamin D Receptor (VDR)

The vitamin D receptor (VDR, NR1I1) is a 1α-25 dihydroxy vitamin D_3 activated member of the NR superfamily of transcription factors. It plays critical roles in calcium homeostasis, bone development and mineralisation, together with a control of cell growth and differentiation. VDR is expressed in the liver, small intestine, bone, kidney and parathyroid gland, and is involved in the up-regulation of the drug metabolising genes CYP2B6, CYP2C9, CYP3A4, CYP24A1 and SULT2A1 (Martinez-Jimenez *et al.*, 2007; Pascussi *et al.*, 2008; Tirona *et al.*, 2005). VDR is also implicated in the increased expression of the drug transporter ABCC3 in the intestine (Makishima *et al.*, 2002; Pascussi *et al.*, 2008).

VDR forms non-permissive heterodimers with RXR (Gronemeyer *et al.*, 2004a). VDR-RXR heterodimers bind to hexameric REs (5'-AG(G/T)TCA-3') arranged as a DR3 in the distal enhancer region, DR4, ER6 (in the PXRE ER6 motif of the CYP3A4 proximal promoter), ER6 (5'TGAACTcaaagg-AGGTCA-3') in the VDRRE proximal promoter region or IR (Makishima *et al.*, 2002; McKenna *et al.*, 1999; Pascussi *et al.*, 2008).

Bile acids including lithocholic acid are VDR agonists, and have been suggested to regulate intestinal CYP3A4 expression (Urquhart *et al.*, 2007). An observation that 1α-25 dihydroxy vitamin D_3-induced CYP3A4 expression in intestinal Caco-2 cells indicated that VDR regulated this drug metabolising enzyme (Bodin *et al.*, 2005; Thummel *et al.*, 2001). Given the co-expression of VDR and CYP3A4 in enterocytes, dietary vitamin D may modulate intestinal first-pass drug metabolism.

PXR, CAR and VDR share REs which initiate a mechanism for NR crosstalk. The consequences of this crosstalk surround the catabolism of vitamin D hormones by CYP3A4 leading to decreased levels of vitamin D and its metabolites together with a disruption of VDR target genes by xenobiotics (Pascussi *et al.*, 2008). The deleterious effects of long-term drug therapy on vitamin D blood levels and bone mineralisation have recently been reported (Pascussi *et al.*, 2008; Xu *et al.*, 2006). HAART (especially PIs) has been suspected to play a part in this process as significant bone disorders are common in HIV infected individuals (Fernandez-Rivera *et al.*, 2003). Three mechanisms, which may not be mutually exclusive, have been proposed to explain these long-term therapy effects but further investigation will be required for the proof of concept. The first mechanism proposed involves drug mediated inhibition of vitamin D_3 bioactivation resulting from decreased 25-hydroxylation of vitamin D_3 in the liver. The second proposed mechanism is PXR agonist induced crosstalk between PXR-VDR leading to up-regulation of CYP24. The third potential mechanism involves the inactivation of 1α-25 dihydroxy vitamin D_3 by the induction of intestinal CYP3A4 (Pascussi *et al.*, 2008).

1.6.6 Peroxisome Proliferator Activated Receptor (PPAR)

The peroxisome proliferator activated receptor (PPAR) has three isotypes, α, γ and β/δ. The role of PPARα (NR1C1) in metabolic gene regulation and dyslipidaemia has been investigated (Barbier *et al.*, 2003; Duval *et al.*, 2007). PPARα is activated by a wide range of endogenous and dietary fatty acids (FAs) and their derivatives including prostaglandins, leukotrienes and eicosanoid derivatives of the lipo-oxygenase or cyclo-oxygenase pathways, and non-steroidal anti-inflammatory drugs (NSAIDs) (Francis *et al.*, 2003; Giguere, 1999; Gronemeyer *et al.*, 2004a). PPARs regulate lipid, cholesterol and glucose homeostasis through co-ordination of gene transcription effects in liver, muscle and adipose tissue. PPARs share common ligands but with variable affinity, this together with differential tissue expression and distinct co-activator recruitment preference results in isotype specific effects on target gene expression. PPARα directs lipids to the liver whereas PPARγ initiates lipid storage in peripheral tissues to maintain balance between energy storage and utilisation (Giguere, 1999).

PPARs may regulate metabolic gene expression by the formation of a NR heterodimer with LXRα (Giguere, 1999). PPARs have been implicated in metabolic diseases such as hyperlipidaemia, insulin resistance, coronary artery disease, obesity, diabetes and metabolic syndrome. Metabolic syndrome is characterised by visceral fat accumulation, hyperlipidaemia, elevated triglycerides, elevated blood pressure and hyperglycaemia, symptoms similar to those in HADL (Francis *et al.*, 2003; Giguere, 1999; Villarroya *et al.*, 2007). PPARα directs lipids to the liver where it is involved in energy homeostasis, inflammatory responses and fatty acid catabolism.

PPARα is expressed in the liver (predominant), kidney, heart, skeletal muscle, adipose tissue, monocytes and other metabolically active tissues (Francis *et al.*, 2003; Pascussi *et al.*, 2008).

PPARα enhances the uptake and transport of circulating fatty acids (FAs) through modulation of target genes including apolipoprotein A-I (ApoA-I), fatty acid binding proteins and lipoprotein lipase (LPL). PPARα regulates lipid homeostasis through the above mechanisms (Francis *et al.*, 2003) and bile acid homeostasis through its action on bile acid transport genes including BSEP and Ntcp, and the metabolism genes OATP1 and MDR2 (Tirona *et al.*, 2005). PPARα induction leads to the repression of ApoC-III (Duval *et al.*, 2007; Feldman *et al.*, 2008; Yamada *et al.*, 2007).

Activation of PPARα by dietary polyunsaturated fatty acids (PUFAs) and the fibrate class of drugs has been shown to have beneficial effects on circulating triglyceride levels and plasma lipid profiles. PPARα is involved in energy homeostasis, inflammatory responses and FA catabolism. It is involved in the up-regulation of metabolising enzymes including CYP4A11 and acyl CoA oxidase and is also implicated in the increased expression of UGT1A9, UGT2B4 and ABCB4 (Tirona *et al.*, 2005). Fatty acid β-oxidation also occurs in skeletal muscle but PPARα is not the sole regulator at this site. PPARα also effects gluconeogenesis in the liver and pancreatic islets by promoting the use of pyruvate in gluconeogenesis rather than fatty acid synthesis through up-regulation of pyruvate dehydrogenase kinase 4 (PDK4) (Francis *et al.*, 2003; Giguere, 1999).

SREBP-1c is a natural stimulatory ligand of PPARγ (NR1C3) and is thus an inducer of PPARγ mediated adipogenesis and adiponectin production (Lee *et al.*, 2003). PIs have been shown to inhibit SREBP-1c and impact PPARγ mediated glucose and lipid metabolism (Bastard *et al.*, 2002; Telenti *et al.*, 2002). At the molecular level, adiponectin expression is stimulated by the PPARγ/SREBP-1c pathway. Adiponectin acts by enhancing the suppression of insulin-induced hepatic gluconeogenesis whilst increasing FFA β-oxidation and insulin-dependent transport in muscle (Iwaki *et al.*, 2003). Low levels of adiponectin and its mRNA expression have been shown in conditions associated with insulin resistance and fat redistribution, including HADL (Haque *et al.*, 2002). The effect of RTV on PPARγ has been associated with decreased adipogenesis, lipogenesis and increased lipolysis, which together with a decrease in adipocyte LPL mRNA levels have been implicated in the dyslipidaemia and insulin resistance mechanisms of HADL (Carr, 2000; Lenhard *et al.*, 2000).

PPARγ is ubiquitously expressed and has three isoforms PPARγ1, PPARγ2 and PPARγ3 (Francis *et al.*, 2003; Pascussi *et al.*, 2008). PPARγ1 is expressed in adipose tissue (predominant), large intestine and haematopoietic cells (low levels are found in kidney, liver, pancreas, small intestine, skeletal and smooth muscle), PPARγ2 expression is restricted to white adipose tissue (where it comprises 30% of total PPARγ), PPARγ3 is expressed in the large intestine and macrophages (Francis *et al.*, 2003; Pascussi *et al.*, 2008). PPARγ is involved in energy homeostasis, inflammatory responses, adipogenesis and the storage of fatty acids (Francis *et al.*, 2003; Giguere, 1999). PPARγ is involved in the up-regulation of CYP4B1 and is also implicated in the increased expression of ABCC1 (Tirona *et al.*, 2005).

PPARγ is a major regulator of adipocyte differentiation, the subsequent uptake and storage of lipids in the mature adipocyte, and a major regulator of insulin sensitivity. Activation of PPARγ promotes cell cycle arrest through the induction of cell cycle inhibitors together with the repression of cyclins and inhibitors of cyclin-dependent kinase. Adipose differentiation related protein, a PPARγ target gene, is up-regulated during pre-adipocyte to mature adipocyte differentiation and is essential for the maintenance of lipid droplet structure (Francis *et al.*, 2003; Khan *et al.*, 2003).

PPARδ is ubiquitously expressed and is involved in glucose and lipid metabolism (Fredenrich *et al.*, 2005; Kota *et al.*, 2005). In the liver, PPARδ's major function is the promotion of glycolysis (Lehrke *et al.*, 2005). PPARδ induction is associated with up-regulated FA β-oxidation, increased HDL cholesterol and elevated uncoupling protein (UCP) expression, together with a decrease in TGs (Kota *et al.*, 2005; Lee *et al.*, 2003). Increased HDL cholesterol via PPARδ induction is achieved through its up-regulation of ABCA1 mRNA expression (Takahashi *et al.*, 2007). A single nucleotide polymorphism (SNP), 294T/C, has been identified in exon 4 of the PPARδ gene, which has been associated with elevated LDL cholesterol and decreased HDL cholesterol in hyperlipidaemic males who are homozygous for the rare C allele (Takahashi *et al.*, 2007).

Peroxisome proliferator-activated receptor gamma co-activator-1 alpha (PGC-1α) has been shown to play a critical role in xenobiotic metabolism (Pascussi *et al.*, 2008). It has a key role in cellular energy metabolism through co-ordination of transcriptional programs of mitochondrial biogenesis, adaptive thermogenesis and

FA β-oxidation and is involved in the co-activation of key transcription factors of hepatic gluconeogenesis (Glass, 2006; Pascussi *et al.*, 2008). PGC-1α affects the activity of multiple NRs (including PPARγ, RXRα, FXR, LXRα, HNF4α, PXR and CAR) to fine tune the biological response to ligand receptor interaction and underpin the variability of gene responses to different ligands and metabolic environments (Francis *et al.*, 2003; Gronemeyer *et al.*, 2004a; Handschin *et al.*, 2003; Pascussi *et al.*, 2008; Tirona *et al.*, 2005).

PPARα and PPARγ impact drug disposition genes via the formation of heterodimers with RXR which bind to peroxisome proliferator REs (PPRE) (5'-AGGTCA-3') arranged as a DR1 in the target gene (Francis *et al.*, 2003; Giguere, 1999; Urquhart *et al.*, 2007). PPREs have been in genes controlling all aspects of lipid and carbohydrate metabolism (Giguere, 1999). Ligand binding to PPARs and their dimerisation partner RXR results in permissive transcriptional activation (Tirona *et al.*, 2005). PPARs may regulate gene expression by the formation of a NR heterodimer with LXRα (Giguere, 1999).

1.6.7 Hepatic Nuclear Factor-4α (HNF4α)

Hepatic nuclear factor 4α (HNF4α, NR2A1) is expressed at high levels in the liver, small intestine, pancreas and kidney, but only low levels are found in the testes (Giguere, 1999). HNF4α is involved in the up-regulation of the drug metabolising genes CYP2C9, CYP2B6, CYP2A6, CYP2D6, CYP3A1, CYP3A16, CYP3A23, CYP3A4 and CYP7A1. HNF4α is also implicated in the increased expression of ABCB1, PXR, CAR, FXR and PPARα (Pascussi *et al.*, 2008; Tirona *et al.*, 2005).

HNF4α is a member of the NR superfamily critical to the maintenance of hepatic phenotype. It functions as a homodimer to DR1-HREs and is constitutively active through its binding of integral fatty acids. It regulates the expression of genes involved in cholesterol, xenobiotic, carbohydrate, lipid and amino acid metabolism (Giguere, 1999). HNF4α is a critical regulator in the constitutive expression of CYP genes. HNF4α constitutive induction is achieved through interactions with steroid hormone co-activators and p300, indicating that an endogenous ligand found in most cell types regulates its activity (Giguere, 1999). Induction of gene transcription by PXR and LXR appears to require HNF4α activity (Tirona *et al.*, 2003; Urquhart *et al.*, 2007).

1.6.8 Glucocorticoid Receptor (GR)

The glucocorticoid receptor (GR, NR3C1) has ubiquitous tissue expression and is involved in the up-regulation of the clinically important drug metabolising genes CYP2C9, CYP2B6, CYP3A4 and CYP3A5. GR is also implicated in the increased expression of PXR, FXR and CAR (Tirona *et al.*, 2005). A detailed review of GR activation has not been included in this research.

1.7 Aims of the Thesis

The specific objectives of this research were two-fold. The first objective was to determine direct effects of the PIs RTV and ATV on the metabolic NRs with a specific focus on the role of FXR, LXRα and PPARα in the metabolic complications associated with the use of these drugs. NR effects were determined by *in vitro* methods which measured both the effects on specific and downstream target gene expression, and the differences in cellular transport of each PI. The effects on gene

expression were further evaluated by evaluating the effects of antagonism and knockdown by small interfering RNA (siRNA) on gene expression. The second objective was to determine whether or not polymorphisms previously associated with metabolic complications in non-HIV infected cohorts influenced the changes in lipid outcomes of an Italian HIV patient cohort which had been switched from a boosted ATV regimen to an unboosted ATV regimen.

CHAPTER 2

THE IMPACT OF A LIVER OXYSTEROL RECEPTOR ALPHA (LXRA) ANTAGONIST ON THE GENE EXPRESSION PROFILES OF RITONAVIR AND ATAZANAVIR IN HEPATIC CELLS

2.1 Introduction

Nuclear receptors (NRs) are present in many tissues of the body including hepatocytes (Tirona *et al.*, 2005; Urquhart *et al.*, 2007). The protease inhibitors (PIs) ritonavir (RTV) and atazanavir (ATV) have been shown to be substrates for/activators of the NRs including the pregnane xenobiotic activated receptor (PXR) and the constitutive androstane receptor (CAR) (Dussault *et al.*, 2001; Healan-Greenberg *et al.*, 2008; Lehmann *et al.*, 1998). PIs have also been shown to impact several molecular pathways key to lipid metabolism including intra-nuclear transcription factors and the nuclear proteasome (Lagathu *et al.*, 2005; Mallon, 2007). Previous studies have focused on the roles of PXR and CAR in the metabolism of RTV and ATV with limited investigation of the role of the metabolic NRs controlling lipid and bile homeostasis in response to PI exposure.

The farnesoid X activated receptor (also known as the farnesoid X receptor, FXR) and the liver oxysterol activated receptor alpha (also referred to as the Liver oxysterol receptor alpha, LXRα) are master regulators of cholesterol homeostasis, and modulate the transcription of many key genes involved in drug and lipid metabolism including the sterol regulatory element binding protein-1c (SREBP-1c), the most abundant sterol regulatory binding protein (SREB) isoform in adult human liver and a key player in lipogenesis (Hui, 2003; Liang *et al.*, 2001; Osborne, 2000). Details of the regulatory mechanisms and target genes for the metabolic NRs are outlined in Section 1.4 of this thesis. The complex nature of the metabolic NRs involved in bile acid metabolism and detoxification together with genes and transporters regulated by the activation of the NRs is outlined in Figure 2.1, below.

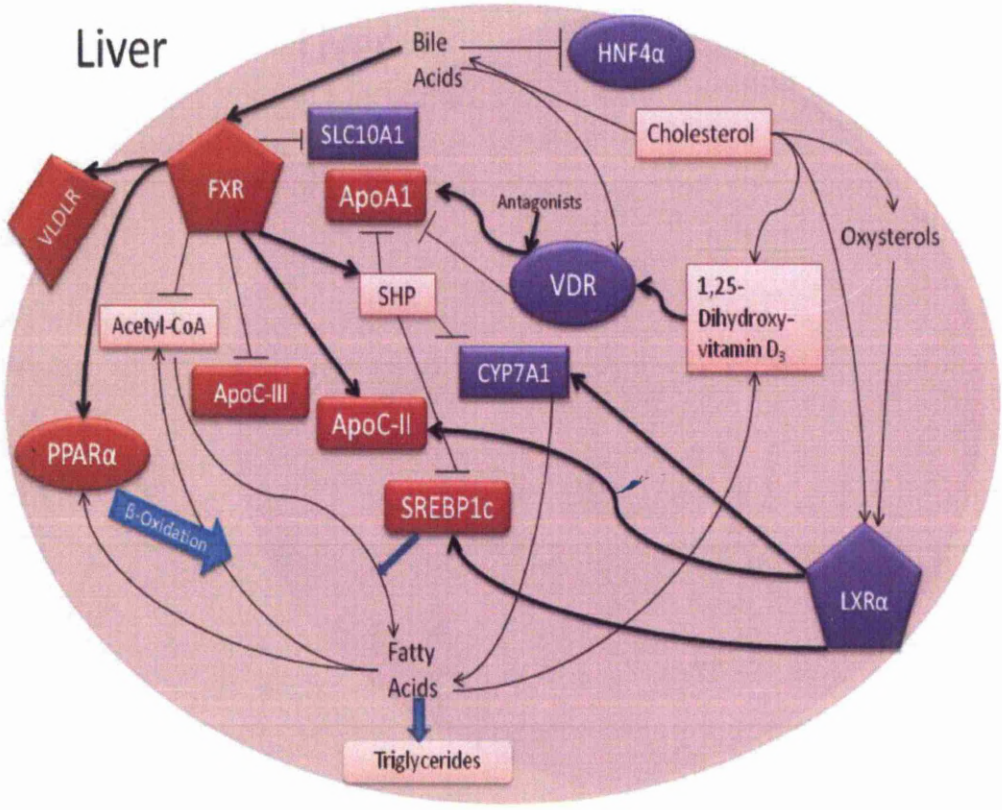


Figure 2.1: Metabolic NRs involved in bile acid metabolism: Arrows = transcriptional up-regulation, barred lines = transcriptional suppression.

RTV is no longer used as a PI in highly active antiretroviral therapy (HAART) but it is currently used at low dose to boost the activity of other PIs through its inhibition of the cytochrome P450 (CYP) enzyme, CYP3A4. RTV has no intrinsic antiviral activity at low dose but it has been widely implicated in the aetiology of hypertriglyceridaemia (Kim *et al.*, 2006b; Mallon, 2007; Purnell *et al.*, 2000). The pathogenesis of the RTV-induced effects has been linked to the effect of RTV on hepatic and adipocyte SREBs including SREBP-1c (Hui, 2003; Liang *et al.*, 2001; Osborne, 2000). RTV has also been associated with enhancing the formation of very

low density lipoproteins (VLDL). A study in the human adipocyte cell line, Simpson Golabi Behmel Syndrome (SGBS), and clinical trials have shown that the newer PI ATV is associated with fewer signs of the deleterious metabolic side effects linked to lipodystrophy (Barreiro *et al.*, 2005; Flammer *et al.*, 2009; Fuster *et al.*, 2005; Kim *et al.*, 2006b).

The direct toxicity attributed to long-term HAART together with the complex interactions with human immunodeficiency virus (HIV) infection and possible other factors, (e.g. genetic predisposition, environmental) influence the risk of developing HIV/HAART associated dyslipidaemic lipodystrophy (HADL). Successful regimens have greatly improved morbidity and mortality outcomes for HIV+ individuals but the onset of HADL greatly increases the risk of developing cardiovascular disease and associated metabolic complications (Kim *et al.*, 2006b; Mallon, 2007). The development of newer antiretrovirals (ARVs) which both overcome the problem of viral resistance and exhibit improved safety profiles for long-term use in an aging HIV+ population is particularly important.

LXRα is highly expressed in the liver and is a regulator of cholesterol homeostasis in the liver and macrophages (Gronemeyer *et al.*, 2004a; Kalaany *et al.*, 2006; Pascussi *et al.*, 2008). It facilitates the processing and storage of cholesterol by up-regulating the cytochrome P450 7A1 enzyme (CYP7A1) and is implicated in the increased expression of the ATP binding cassette proteins ABCA1, ABCG1, ABCG5 and ABCG8 (Eloranta *et al.*, 2005; Pascussi *et al.*, 2008; Tirona *et al.*, 2005). The transcriptional activity of LXRα is induced by elevated levels of naturally occurring

oxysterols and 6α-hydroxy bile acids (Eloranta *et al.*, 2005; Francis *et al.*, 2003). LXRα is also abundant in adipose tissue, where its activation has been shown to increase basal glucose uptake and the incorporation of TGs into lipid droplets (Lehrke *et al.*, 2005).

LXRα responds to high cholesterol levels by increasing fatty acid synthesis to increase the formation of cholesterol esters, rendering the excess cholesterol pool biologically inactive (Giguere, 1999; Pascussi *et al.*, 2008; Urquhart *et al.*, 2007). LXRα also facilitates efflux of cholesterol from the periphery, including macrophages, through the increased expression of ABC transporters. Induction of ABC transporters increases the transfer of cholesterol and phospholipids to apolipoprotein A-I (ApoA-I) containing lipoproteins. Furthermore, activation of LXRα decreases intestinal absorption of cholesterol mediated through the induction of ABC transporters including ABCG5 and ABCG8 (Kalaany *et al.*, 2006; Pascussi *et al.*, 2008). The effects of LXRα on fatty acid synthesis are mediated through the increased expression of SREBP-1c, a stimulator of lipogenesis. SREBP-1c is a transcription factor which has been previously associated with PI induced lipodystrophy (Bastard *et al.*, 2002; Telenti *et al.*, 2002). Increased production of liver triglycerides alongside transient increases in plasma levels resulting from the up-regulation of SREBP-1c remains a hurdle to the development of therapeutic LXRα agonists.

FXR, the counterbalance of hepatic LXRα action, is activated by bile acid accumulation resulting from the LXRα mediated conversion of cholesterol to bile

acids. High concentrations of bile acids can be toxic to the cell and FXR functions to minimise this by inhibiting further bile acid production. Inhibition of bile acid synthesis is achieved by FXR down regulating CYP7A1, the rate-limiting enzyme in bile acid synthesis, through indirect signalling involving the increased expression of the small heterodimer partner-1 (SHP-1)-liver receptor homologue-1 (LRH-1) pathway. SHP-1 represses CYP7A1 expression by binding to regulatory sequences and preventing further response element (RE) binding of activated LXRα (Eloranta *et al.*, 2005).

PPARs may regulate metabolic gene expression by the formation of a NR heterodimer with LXRα (Giguere, 1999). PPARs have been implicated in metabolic diseases such as hyperlipidaemia, insulin resistance, coronary artery disease, obesity, diabetes and the metabolic syndrome (Behrens *et al.*, 2000; Blaschke *et al.*, 2006; Duval *et al.*, 2007; Lee *et al.*, 2003). Metabolic syndrome is characterised by visceral fat accumulation, hyperlipidaemia, elevated triglycerides, elevated blood pressure and hyperglycaemia, symptoms similar to those in HADL (Francis *et al.*, 2003; Giguere, 1999; Villarroya *et al.*, 2007). PPARα directs lipids to the liver where it is involved in energy homeostasis, inflammatory responses and fatty acid catabolism. PPARα is expressed in the liver, kidney, heart, skeletal muscle, adipose tissue, monocytes and other metabolically active tissues (Francis *et al.*, 2003; Pascussi *et al.*, 2008).

PPARα is activated by a wide range of endogenous and dietary fatty acids (FAs) and their derivatives including prostaglandins, leukotrienes and eicosanoid derivatives of

the lipo-oxygenase or cyclo-oxygenase pathways, and non-steroidal anti-inflammatory drugs (NSAIDs) (Francis *et al.*, 2003; Giguere, 1999; Gronemeyer *et al.*, 2004a). PPARα enhances the uptake and transport of circulating fatty acids (FAs) through modulation of its target genes including apolipoprotein A-I (ApoA-I), fatty acid binding proteins and lipoprotein lipase (LPL) (Berger *et al.*, 2002; Lee *et al.*, 2003). PPARα activation by dietary polyunsaturated fatty acids (PUFAs) and fibrates has been shown to have positive effects on circulating triglyceride levels and plasma lipid profiles (Adkins *et al.*; Clarke, 2001; Tobin *et al.*, 2006).

SREBP-1c is a natural stimulatory ligand of PPARγ and is thus an inducer of PPARγ mediated adipogenesis and adiponectin production (Lee *et al.*, 2003). PIs have been shown to inhibit SREBP-1c and impact PPARγ mediated glucose and lipid metabolism (Bastard *et al.*, 2002; Telenti *et al.*, 2002). At the molecular level, adiponectin expression is stimulated by the PPARγ/SREBP-1c pathway. Adiponectin acts by enhancing the suppression of insulin-induced hepatic gluconeogenesis whilst increasing FFA β-oxidation and insulin-dependent transport in muscle (Iwaki *et al.*, 2003). Low levels of adiponectin and its mRNA expression have been shown in conditions associated with insulin resistance and fat redistribution, including HADL (Haque *et al.*, 2002). The effect of RTV on PPARγ has been associated with decreased adipogenesis, lipogenesis and increased lipolysis, which together with a decrease in adipocyte LPL mRNA levels have been implicated in the dyslipidaemia and insulin resistance mechanisms of HADL (Carr, 2000; Lenhard *et al.*, 2000). The implications of FXR, LXRα, PPARα and PPARγ in metabolic complications are summarised in Table 2.1 below.

Peroxisome proliferator-activated receptor gamma co-activator-1 alpha (PGC-1α) has been shown to play a critical role in xenobiotic metabolism (Pascussi *et al.*, 2008). It has a key role in cellular energy metabolism through co-ordination of transcriptional programs of mitochondrial biogenesis, adaptive thermogenesis and FA β-oxidation and is involved in the co-activation of key transcription factors of hepatic gluconeogenesis (Glass, 2006; Pascussi *et al.*, 2008). PGC-1α affects the activity of multiple NRs (including PPARγ, RXRα, FXR, LXRα, HNF4α, PXR and CAR) to fine tune the biological response to ligand receptor interaction and underpin the variability of gene responses to different ligands and metabolic environments (Francis *et al.*, 2003; Gronemeyer *et al.*, 2004a; Handschin *et al.*, 2003; Pascussi *et al.*, 2008; Tirona *et al.*, 2005).

Table 2.1: Summary of NRs implicated in metabolic complications and their effects

FXR	**LXRα**	**PPARα**	**PPARγ**
↑↑HDL	↑↑HDL	↑HDL	
↓LDL	↓LDL		
↓TGs	↓TGs	↓TGs	
		↓VLDL	
↓ Insulin resistance	↓ Insulin resistance		↓ Insulin resistance
	↑Fat	↓FFAs	↑Fat
↑Reverse cholesterol transport	↑Reverse cholesterol transport	↑Reverse cholesterol transport	↑Reverse cholesterol transport

The Huh7 cell line is an immortal cell line of epithelial-like tumorigenic cells. The Huh7 is a well differentiated cell line derived from a human carcinoma cell line

originally derived from a hepatic tumour in a 57 year old Japanese male in 1982. Previous studies by the Liverpool HIV Pharmacology Group had considered the limitations of cell lines compared to primary hepatocytes and the implications of discrepancies reported between laboratories (Martin *et al.*, 2007). Due consideration was given to the findings of this study when considering experimental design for the hepatic cell line model and comparative gene expression for the genes of interest was investigated prior to final selection of the preferred cell line, see Section 2.3.2 below. A stable cell line which could be sub-cultured in antibiotic free media was preferred. Ease of culture and reproducibility of data obtained from the immortalised cell lines favoured their use over primary hepatocytes. The Huh7 cell line was selected as the preferred experimental system for the gene expression studies as experience within the laboratory had revealed a better cell health profile for repeated sub-cultures in antibiotic free media compared to the widely used human hepatoma cell line HepG2.

Target genes for this thesis were selected on the basis of their involvement in fatty acid, cholesterol, lipid or triglyceride metabolism and based on previous reports as being regulated by the metabolic nuclear receptors FXR, LXRα and PPARα (Table 2.2, below). PXR, CAR and PPARγ were excluded as they have previously been extensively investigated (Feldman *et al.*, 2008; Guo *et al.*, 2006; Healan-Greenberg *et al.*, 2008; Kodama *et al.*, 2004; Lemoine *et al.*, 2006).

RTV was selected on the basis of its widespread use as a booster of PI activity in HAART and its previously reported involvement with the metabolic complications associated with PIs (Falutz, 2007; Kim *et al.*, 2006a; Mallon, 2007; Purnell *et al.*,

2000). ATV was selected on the basis of it being a newer PI with fewer metabolic side effects (Kim *et al.*, 2006b). 22-(S)-hydroxycholesterol has previously been shown to bind LXRα without activation (Janowski *et al.*, 1999) and was therefore considered a suitable LXRα antagonist to probe the involvement of LXRα in the metabolic NR response to RTV.

Table 2.2: Human FXR, LXRα and PPARα target genes with their endogenous biological metabolic function and effects of induction shown.

Gene Name	Abbrev. Name	Biological Function	Regulation by FXR	Regulation by LXRα	Regulation by PPARα
Fatty acid transport protein	FATP	Fatty acid metabolism			Induced
Fatty acid translocase	FAT	Fatty acid metabolism			Induced
Apolipoprotein C-III	ApoC-III	TG metabolism	Repressed	Induced (via PPARα repression)	Repressed
Apolipoprotein A1	ApoA-I	HDL metabolism	Repressed	Induced or Repressed	Induced
Lipoprotein lipase	LPL	TG metabolism	Induced (via ApoC-II induction)	Induced	Induced
Apolipoprotein C-II	ApoC-II	TG metabolism	Induced	Induced	Repressed
Apolipoprotein A2	ApoA2	HDL metabolism			Induced
Apolipoprotein A5	ApoA5	HDL metabolism			Induced
Fatty acid synthase	FAS	Fatty acid metabolism	Induced or Repressed	Induced	Induced
Sterol Regulatory Element Binding Protein-1c	SREBP-1c	Lipogenesis	Repressed (via SHP-1 induction)	Induced	
Very Low Density Lipoprotein Receptor	VLDLR	TG metabolism	Induced	Repressed	Induced
Cytochrome P450 7A1	CYP7A1	Cholesterol metabolism	Repressed (via SHP-1 induction)	Induced	Repressed
Solute Carrier Family 10 A1	SLC10A1	Cholesterol and bile acid metabolism	Repressed		Repressed
Peroxisome Proliferator Activated Receptor-α	PPARα	TG metabolism	Induced	Repressed	

2.2 Aim

The aims of this research were to investigate the specific impact of the PIs RTV and ATV on gene expression in NRs involved in lipid metabolism identified in Section 2.1 of this thesis, and on the gene expression of downstream targets in their metabolic pathways (Table 2.2); to establish if the differential effects in gene expression in the presence of RTV and ATV could be attributed to differences in the cellular accumulation of these drugs; and to determine the effect of an LXRα antagonist (22-(S)-hydroxycholesterol) on gene expression in RTV and ATV treated cells.

2.3 Materials and Methods

2.3.1 Materials

Normal liver RNA, normal intestinal RNA and the Huh7 cell line were obtained from the European Collection of Cell Cultures (ECACC) (Salisbury, UK). HepG2 and Caco-2 cell lines were obtained from American Type Culture Collection (Manassas, USA). Forward and reverse primers for in-house developed assays (HSP70, VDR, IBABP-L, and HNF4α), Molecular Grade Ethanol, Molecular Grade Isopropanol, Molecular Grade Methanol, Sodium Dodecyl Sulphate (SDS), N_1,N_1-Dmimethylformamide (DMF), Thiazolyl Blue Tetrazolium Bromide, Hanks Balanced Salt Solution (HBSS), Dimethylsulphoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), RNAse free water, DNAse free water, newborn foetal calf serum (FCS), foetal bovine serum (FBS), Trypsin-EDTA x1, 2-mercaptoethanol, Chloroform, potassium chloride, HEPES free acid, magnesium chloride, potassium hydroxide pellets, and Tri®Reagent RNA isolation reagent were purchased from Sigma Aldrich Co. Limited (Irvine, Ayrshire, UK). 10x Taqman PCR Buffer II,

25mM $MgCl_2$, 5U/µl Amplitaq Gold DNA Polymerase, 100mM dNTPs, Taqman Reverse Transcription Reagents, Taqman Gene Expression inventoried Assays and Taqman Endogenous Control Gene Expression Plates were purchased from Applied Biosystems Limited (Warrington, UK). Absolute qPCR mix was purchased from ABgene Limited (Epsom, Surrey, UK). Nunc cell culture flasks, Nunclon Surface 96 well plates, Nunclon Surface 48 well plates, Nunclon Surface 6 well plates and Nunclon Up-Cell 6 well plates were purchased from (Nunc A/S, Kamstrup, Denmark). PicoGreen dsDNA Quantitation Reagent was purchased from Invitrogen Limited (Paisley, UK). DNA Molecular Weight Marker XIV (100bp ladder) was purchased from Roche Diagnostics Limited (Burgess Hill, UK). Ritonavir and Radiolabelled ritonavir (^{14}C, 99.9% estimated purity) were kind gifts from Abbott Laboratories (North Chicago, USA). Atazanavir was a kind gift from Bristol-Myers Squibb (USA). Darunavir was a kind gift from Tibotec BVBA (Beerse, Belgium). Indinavir was a kind gift from Merck and Co. (New Jersey, USA). Radiolabelled atazanavir (3H, >99% estimated purity) was purchased from Moravek Biochemicals (California, USA). Ultima Gold Scintillation Fluid was purchased from Perkinn Elmer, (Boston USA). The DNA Engine Opticon 2 system was from MJ Research Inc. (USA).

2.3.2 Cells and Cell Maintenance

2.3.2.1 Huh7, HepG2 and Caco-2 Cell Lines

Huh7 and HepG2 cells were maintained in DMEM supplemented with 10% FBS, incubated at 37°C and 5% CO_2 and were sub-cultured every 2–3 days. Basal levels of all transcripts were quantified from passages 14–32 in Huh7 cells and from passages 16-20 in HepG2 cells.

Caco-2 cells were maintained in DMEM supplemented with 15% FCS, incubated at 37°C and 5% CO_2 and were sub-cultured every 2–3 days. Basal levels of all transcripts were quantified from passages 28–52 in Caco-2 cells.

2.3.2.2 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Toxicity Assays

RTV, ATV, indinavir (IDV) and darunavir (DRV) were assayed for toxicity in Huh7 by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at final concentrations of 0.01–100μM in DMSO against DMSO-treated controls, and at final concentrations of 0.01–100μM in methanol against methanol-treated controls at 0.5% v/v for vehicles. The toxicity of 22-(S)-hydroxycholesterol was also determined by the MTT assay at final concentrations of 0.01–100μM in methanol against methanol-treated controls at 0.5% v/v for vehicles. Incubation was for 120h at 37°C and 5% CO_2 prior to assessment of cell death by fluorescence at 560nm via a GENios XFLUO4 plate reader. Methanol was determined to be the preferred vehicle for toxicity and induction assays (see section 2.5.1). Drugs were determined to be toxic where the cell viability after 120h was below 85% compared to the untreated cells.

2.3.2.3 Cell Treatment

Cell lines were seeded at a density of $5x10^6$ per well into 10% FCS-supplemented DMEM medium, in Nunclon Surface 6 well plates and incubated at 37°C and 5% CO_2 for 24h prior to the set-up of all expression experiments. The concentration–response experiments for RTV and ATV were conducted at final concentrations of 0, 0.1, 1.0, and 10μM, determined by cell viability after 120h toxicity assays.

Methanol-treated controls were used (0.5% v/v for vehicles). RTV and ATV treated Huh7 cells were incubated at 37°C and 5% CO_2 and were sampled at 24h. The concentration–response experiments for RTV plus 22-(S)-hydroxycholesterol treated Huh7 cells were conducted at final concentrations of 0, 0.1, 1.0, and 10μM RTV plus a fixed concentration of 20μM 22-(S)-hydroxycholesterol in each assay. The cells were incubated at 37°C and 5% CO_2 and were sampled at 24h.

2.3.2.4 RNA Isolation from Huh7 Cells

Isolation of RNA from untreated, RTV-treated, ATV-treated, and RTV plus 22-(S)-hydroxycholesterol-treated Huh7 cells was undertaken using the Tri®Reagent method in accordance with the manufacturer's instructions. Briefly, cells were lysed directly from plate in Tri-Reagent by repetitive pipetting (1ml Tri/5-10x10^6 cells) and incubated for 5 minutes at room temperature prior to transfer to Eppendorf® tubes. 200ul of chloroform were added to each tube. Each tube was shaken vigorously and incubated at room temperature for 2-3minutes. Samples were centrifuged at 12000 x g for 15 minutes at 2°C. The upper aqueous phase was transferred to a fresh tube, 500ul isopropanol added, incubated at room temperature for 10 minutes, then centrifuged at 12000 x g for 10 minutes at 2°C. The supernatant was discarded, 1ml of 75% ethanol was added, tubes were vortexed and centrifuged at 7500 x g for 5 minutes at 2°C. The supernatant was removed and the pellet air-dried for 10minutes. The RNA pellets were dissolved in 20μl ddH_2O and incubated for 10 minutes at 60°C. The samples were analysed and quantified using a Thermo-Scientific® Nanodrop 1000 to assess quantity and purity of the RNA extracted. RNA is detected at 260nm (where 40μg/ml corresponds to an optical density [OD] of 1.00) and

protein at 280nm, an A_{260}/A_{280} ratio of over 1.8 indicated samples of sufficient purity to proceed with PCR.

2.3.2.5 Production of cDNA

RNA was isolated as described above (2.2.2.4) and cDNA was synthesised in a total reaction volume of 50µl with the total RNA normalised to 2µg per 50µl reaction. The RNA to cDNA reverse transcription reactions were performed in 5µl of 10x Taqman PCR Buffer II, 11µl of 25mM $MgCl_2$, 10µl of 10mM dNTPs, 2.5µl of 50µM random hexamers, 1µl of 20U/µl RNase inhibitor, 1.75µl of 50U/µl MuLV reverse transcriptase (Applied Biosystems Limited, Warrington, UK) and RNase free water up to 50µl volume. Reverse transcription was undertaken with the following cycling conditions: 10 minutes at 25°C, 30 minutes at 48°C and, 5 minutes at 95°C. Samples were analysed and quantified using a Thermo-Scientific® Nanodrop 1000 to assess quantity and purity of the cDNA.

DNA is detected at 260nm (where 40µg/ml corresponds to an optical density [OD] of 1.00) and protein at 280nm, an A_{260}/A_{280} ratio of over 1.7 indicated samples of sufficient purity to proceed with PCR. The concentration of each sample was normalised to 20ng/µl by the addition of DNAse free water.

Normal liver RNA, normal intestinal RNA (European Collection of Cell Cultures (ECACC) Salisbury, UK) was also reverse transcribed using the Taqman Reverse Transcription kit, analysed and quantified as described above.

2.3.2.6 Cellular Accumulation of Ritonavir and Atazanavir

In order to assess the intracellular accumulation of RTV and ATV, Huh7 cells at passage 14 were resuspended in media containing [^{3}H]-ATV (0.02μCi·ml^{-1}) or [^{14}C]-RTV (0.02μCi·mL^{-1}). The cells were incubated at 37°C/5% CO_2 for 30 minutes, centrifuged at 9000rpm for 1 minute at 2°C. 100μl aliquots of each supernatant fraction were transferred to corresponding scintillation vials. The remaining supernatant was discarded and the pellets were washed three times in ice cold Hanks Balanced Salt Solution (HBSS), centrifuged at 9000rpm for 1 minute at 2°C and then dissolved in 100μl water and transferred to corresponding scintillation vials. Scintillation fluid (4ml) was then added to both supernatant and pellet and the cellular accumulation was calculated (ratio of radiolabelled drug associated with the cell pellets divided by that associated with media). A cell volume of 0.4pl was assumed. The accumulation of ATV and RTV was determined at 0h and 24h post incubation the drugs. Controls consisted of untreated Huh7 incubated in media.

The results of the scintillation count were analysed in MS Excel 2010 by determining the cellular accumulation ratio (CAR) with the following calculation:

CAR = Intracellular associated radioactivity/Extracellular associated radioactivity.

2.3.2.7 Development and Optimisation of In-House Gene Expression Assays

Single-stranded DNA primers were designed in accordance with established protocol laid down by primer manufacturers and the literature (Chou *et al.*, 1992; Jeffreys *et al.*, 1988; Saiki *et al.*, 1988) and were validated *in silico* using the online NCBI Primer-BLAST system (NCBI-Primer-Blast, 2009; Sayers *et al.*; Yu *et al.*).

Briefly, the primer sequences were designed to be 15 to 30 bases in length, did not complement within themselves or to each other; primer concentrations in the range of 0.2mM to 0.5mM were optimised for best results; the concentrations of $MgCl_2$ (Range 1.0 to 4.0mM), dNTPs (Range 0.2 to 0.8mM), DNA polymerase enzyme, annealing temperatures (Range 50°C to 70°C) and the number of PCR amplification cycles (Range 30 -50) were also varied during the optimisation process. Preferred conditions determined by optimisation are outlined in Sections 2.3.2.9 and 2.5.3 below.

The effect of the variations was determined by examining the intensity and distribution of amplification products after electrophoresis on agarose gel followed by visualisation with ethidium bromide staining of the gel.

The in-house developed real time PCR gene expression assays for quantification by Pico-green relative to the expression of the housekeeping gene, β-2-microglobulin (β-2M), were then used to determine the gene expression of HSP70, IBABP-L, VDR and HNF4α. Relative expression of the transcripts against the expression of the β-2M was performed, in duplicate for all assays, in an Opticon2 Fluorescence Detector. Amplification was conducted in a 25µl reaction consisting of: 2.5µl 10 x Taqman Buffer II, 0.5 µl 0.5U/µl Amplitaq Gold, 1.25µl 25mM $MgCl_2$, 2µl 10mM dNTPs, 40ng cDNA (normalised to 20ng/µl), 0.5µl PicoGreen (final concentration 1:5000) and 0.5µl 15µM forward and 0.5µl 15µM reverse primers (for each gene), and nuclease-free water was added to provide a final volume of 25µl. Primer sequences and RT-PCR conditions for in-house developed gene expression assays are outlined in Table 2.4 below.

2.3.2.8 Identification of Controls by Gene Expression Assays in Untreated Cells

Normal liver RNA and normal intestinal RNA was reverse transcribed to cDNA as described previously (Owen *et al.*, 2004) and was normalised to 20ng/µl as described in 2.2.2.5 above. Gene expression was determined in normal liver cDNA, normal intestinal cDNA, and untreated Huh7 cell line cDNA for each assay using the Taqman inventoried gene expression assays (Table 2.3) and in-house developed gene expression assays (Table 2.4) below. Relative expression of each gene was determined by comparative C_T ($\Delta\Delta C_T$) method against the normal liver control values for each experimental condition using Microsoft Office Excel 2010 for analysis.

2.3.2.9 RT-PCR Gene Expression

Taqman Gene Expression inventoried assays for RT-PCR (Applied Biosystems Limited, Warrington, UK, (Table 2.3)) were used for FXR, LXRα, ApoA-I, ApoC-II, ApoC-III, SREBP-1C, VLDLR, SLC10A1, PPARα, CYP7A1, GAPDH and β-actin quantified against the housekeeping gene β2M. Amplification was conducted in a reaction consisting of 25µl Taqman Gene Expression inventoried Assay kit, as supplied. Amplification was performed in an Opticon2 Fluorescence Detector (MJ Research, Bio-Rad, Hertfordshire, UK) at 95°C for 10 minutes then 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. In-house developed real time PCR gene expression assays for quantification relative to β2M (housekeeping gene) were performed on HSP70, IBABP-L, VDR and HNF4α.

Relative expression of the transcripts against the expression of the housekeeping gene was performed, in duplicate for all assays for a minimum of 4 biological

replicates, in an Opticon2 Fluorescence Detector. Amplification was conducted in a reaction consisting of 25µl Taqman Gene Expression inventoried Assay kit, as supplied; and for in-house developed assays: 2.5µl 10 x Taqman Buffer II, 0.5 µl 0.5U/µl Amplitaq Gold, 1.25µl 25mM $MgCl_2$, 2µl 10mM dNTPs, 40ng cDNA (normalised to 20ng/µl), 0.5µl Pico Green (final concentration 1:5000) and 0.5µl 15µM forward and 0.5µl 15µM reverse primers (for each gene), and nuclease-free water was added to provide a final volume of 25µl. Primer sequences and RT-PCR conditions for in-house developed gene expression assays are outlined in Table 2.4 below.

For each gene expression assay, amplification plots were generated by the Opticon 2 software showing the increase in reporter dye fluorescence with each cycle of RT-PCR. The threshold cycle (C_T) values for each assay were determined where the PCR efficiency was at 100% and data was exported to Microsoft Office Excel 2010 for analysis. Calculations for relative mean gene expression were performed using the comparative (delta-delta) C_T methodology ($\Delta\Delta C_T$) (Bookout *et al.*, 2003). Expression values were normalised against the mean expression of the relative housekeeping gene and plotted against vehicle controls for each gene.

Table 2.3: Taqman Gene Expression Assays for RT PCR

Taqman Assay ID	Assay Gene
Hs00231968_m1	FXR
Hs00231882_m1	PPARα
Hs00167982_m1	CYP7A1
Hs01088691_m1	SREBP-1c
Hs03037356_m1	APOC2 (ApoC-II)
Hs00163644_m1	APOC3 (ApoC-III)
Hs01045922_m1	VLDLR
Hs00161820_m1	SLC10A1 (NTCP)
Hs00163641_m1	APOA-I (ApoA-I)
Hs00172885_m1	LXRα
Hs99999907_m1	β2M
Hs99999903_m1	β-actin
Hs99999905_m1	GAPDH

2.4. Statistical Analysis

Statistical analysis of all results was undertaken using StatsDirect Statistical software version 2.7.8. Spearman's Rank tests were performed on the untreated cells to determine correlation between cell line expression and normal tissue gene expression. Shapiro-Wilk W tests were performed to test for normality. Normal distribution of the data sets was confirmed so the Unpaired T-Test was performed on all data sets to determine the significance of the differences in gene expression at varying concentrations. A statistical trend was defined as a P value <0.1. Statistical significance was set at $P<0.05$.

2.5 Results

2.5.1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Toxicity Assays

Results for the DMSO vehicle MTT toxicity assays are not shown as DMSO was rejected as the preferred vehicle due to precipitation of the drug in the DRV and

ATV assays. The MTT toxicity assays using methanol as the drug vehicle for 4 technical and 4 biological replicates were performed for each drug, including 22-(S)-hydroxycholesterol in Huh7 cells. RTV was determined to be toxic in Huh7 cells (n=4) at 100μM, as shown in figure 2.2 below. IDV was also determined to be toxic in Huh7 cells (n=4) at 100μM, as shown in Figure 2.2 below. DRV was also determined to be toxic in Huh7 cells (n=4) at 10μM and 100μM, as shown in Figure 2.2 below. ATV was determined to be toxic in Huh7 cells (n=4) at 100μM as shown in Figure 2.2 below.

The antagonist 22-(S)-hydroxycholesterol was not found to be toxic in Huh7 cells (n=4) in concentrations up to 100μM, as shown in Figure 2.3 below.

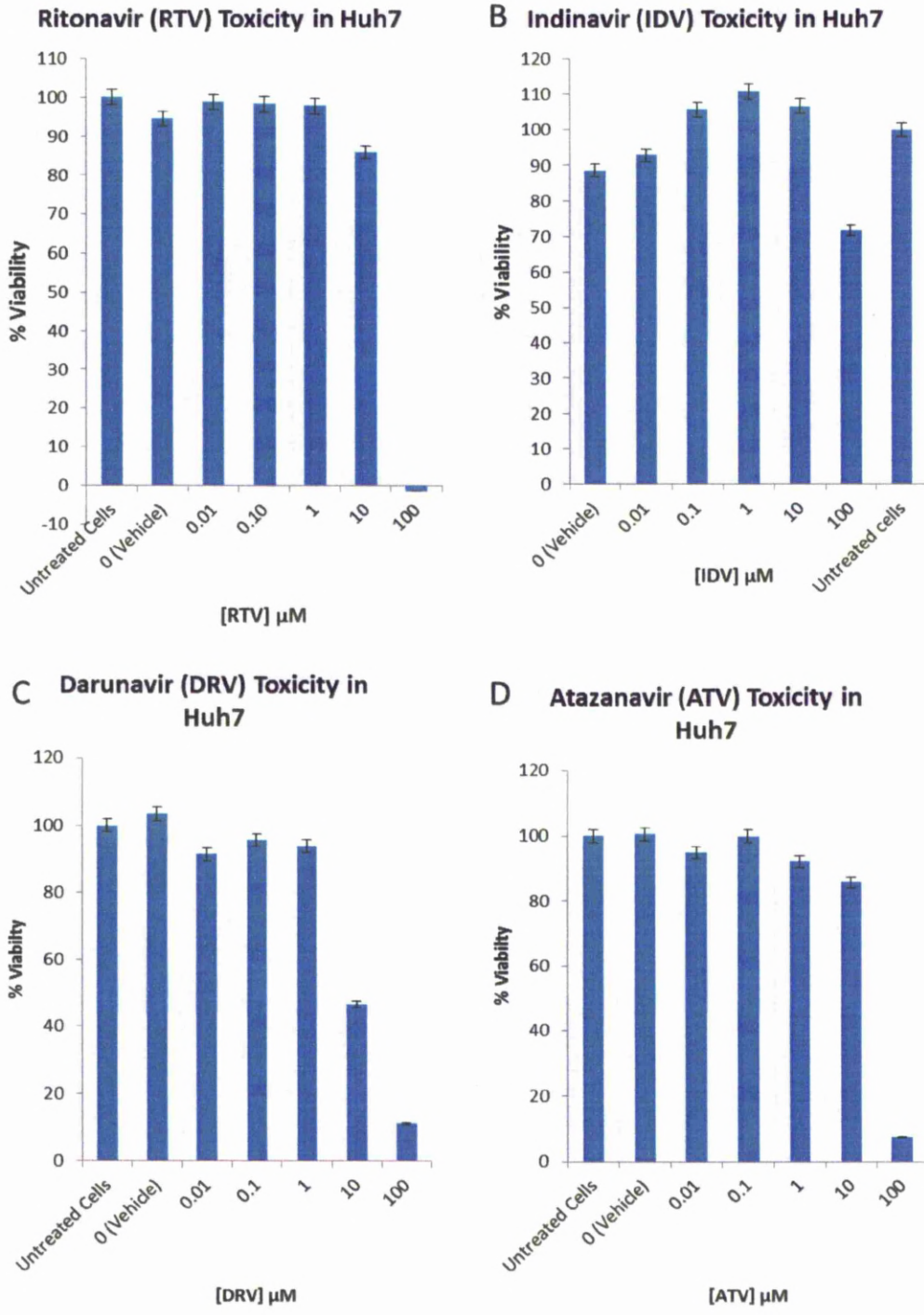


Figure 2.2: MTT Assays in Huh7 (passage =22) Cells: A= Incubated with ritonavir (RTV) for 120h, n=4; B= Incubated with indinavir (IDV) for 120h, n=4; C= Incubated with darunavir (DRV) for 120h, n=4; and D= Incubated with atazanavir (ATV) for 120h, n=4

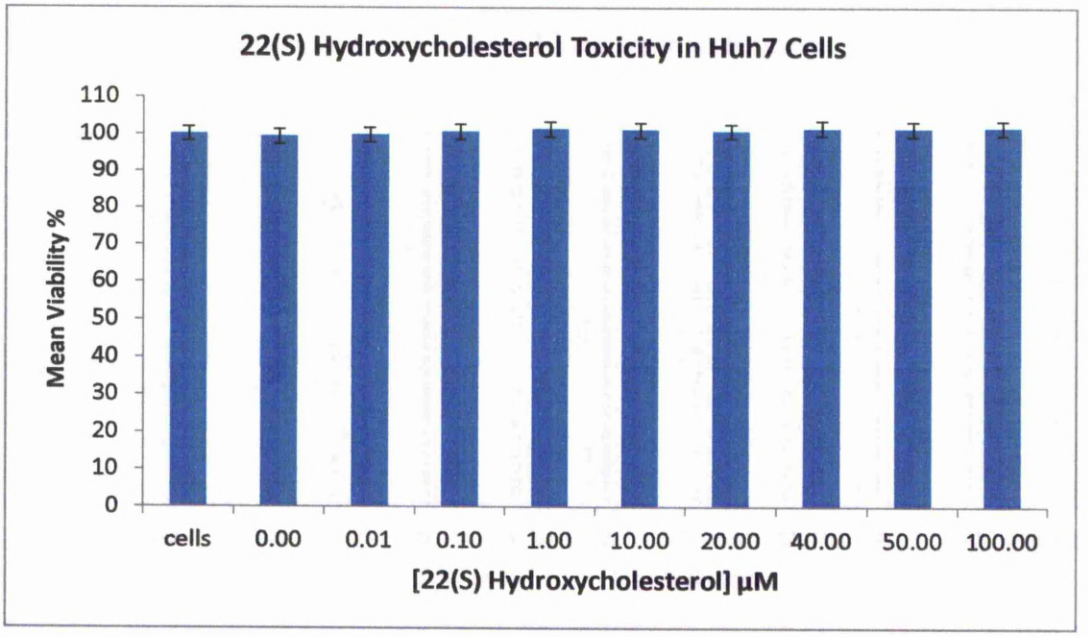


Figure 2.3: MTT Assay: Huh7 cells (passage 22) incubated with 22-(S)-hydroxycholesterol for 120h, n=4

2.5.2 Quantification of RNA and cDNA

The RNA extracted from Huh7 cells (as described in Section 2.3.2.4), was analysed and quantified using a Thermo-Scientific® Nanodrop 1000 to assess its quantity and purity. The RNA had an A_{260}/A_{280} ratio in the range of 1.85-2.01 (RTV samples), 1.92-2.08 (RTV + 22-(S)-hydroxycholesterol samples) and 1.94-2.15 (ATV samples) indicating sufficient purity to proceed to cDNA construction for RT PCR. RNA samples were frozen at -80°C following cDNA construction.

The cDNA for all conditions had A_{260}/A_{280} ratios in the range of 1.98-2.13 indicating sufficient purity to proceed to construction of normalised (20ng/µl) cDNA working stocks. Master and working stock cDNA samples were frozen at -20°C until used in gene expression assays.

2.5.3 Optimisation of In-House Gene Expression Assays

RNA was isolated by the Tri®Reagent method as described in 2.2.2.4 above and reverse transcribed to cDNA as described previously in 2.2.2.5. Single-stranded DNA primers were designed and validation was performed as outlined in Section 2.3.2.7. Each validation reaction contained 40ng cDNA (normalised to 20ng/µl).

Optimum final primer concentrations were determined to be 0.3µM forward and 0.3µM reverse (for each gene) in a final reaction volume of 25µl. The optimum polymerase enzyme was determined as 0.25U/µl Amplitaq Gold per reaction. The optimum final concentrations for the remaining variables outlined in 2.3.2.7 were 1.25mM $MgCl_2$, 0.8mM dNTPs, and 1:5000 PicoGreen (final concentration). Primer sequences and cycle thresholds for annealing and extension in RT-PCR conditions for in-house developed gene expression assays are outlined in Table 2.3 below. The presence of amplification products was confirmed by electrophoresis on agarose gel followed by visualisation of the product by ethidium bromide staining of the gel. Figure 2.4 below shows the validation of VDR gene expression by PicoGreen and the presence of the β-2M and VDR amplicons on the agarose gel, similar validations were performed for HNF4α, HSP70 and IBABP-L (not shown). The gene expression of IBABP-L was below the detection threshold even after 50 cycles, so it was excluded from the research, it was originally included as a potential negative control for the assays, but HSP70 expression was more robust and it was therefore a preferred candidate.

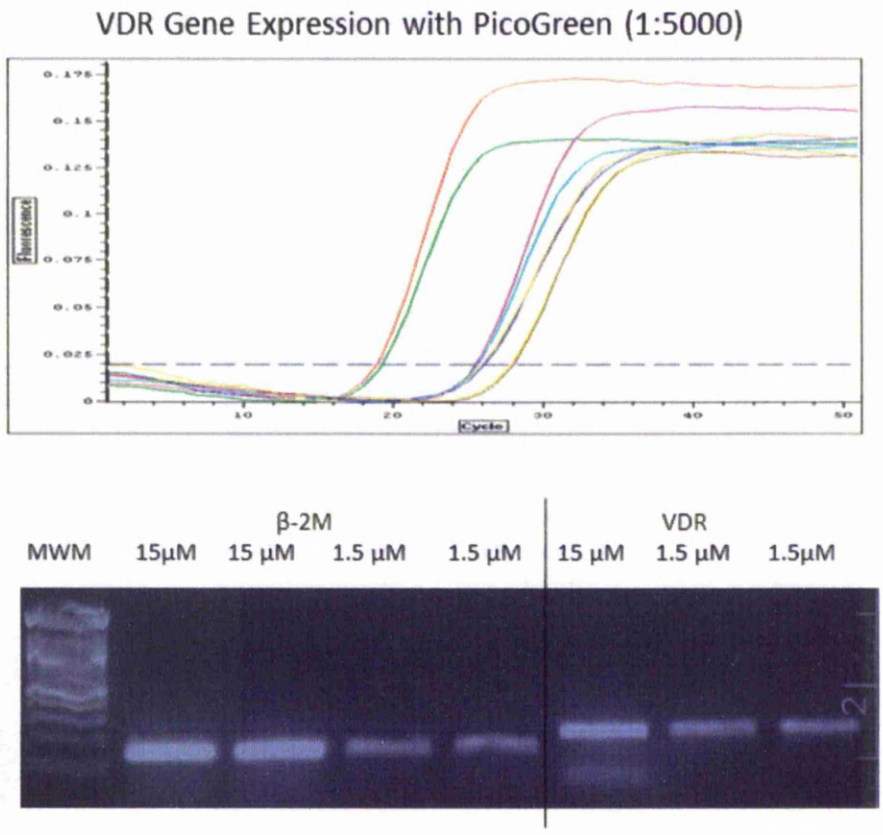


Figure 2.4: Opticon Trace: Representative VDR Gene expression against β-2M control in Huh7 Cells for in-house developed PicoGreen assay, n=2, with validation of amplicon products by electrophoresis. (MWM= Molecular weight marker).

Primer sequences and RT-PCR conditions for in-house developed gene expression assays are outlined in Table 2.4 below.

Table 2.4: In-House Developed Primers for RT PCR Gene Expression Assays Quantified by Pico-green.

Gene Name	Forward Primer 5'-3'	Reverse Primer 5'-3'	RT-PCR Conditions
HNF4α	TTGAGTGGGC CAAGTACATC	TACGGATGGAGTTT CGGTAG	95°C – 10 minutes 40 cycles: 95°C -15 s, 60°C -45 s, 72°C -45 s; 72°C - 5 minutes
VDR	TGCCTGACCCT GGAGACTTTG ACC	CATCATGCCGATGT CCACACAGCG	95°C – 10 minutes 50 cycles: 95°C -20 s, 65°C -30 s, 72°C -45 s; 72°C - 5 minutes
HSP70	CCGAGAAGGA CGAGTTTGAG	GCAGCAAAGTCCTT GAGTCC	95°C – 10 minutes 45 cycles: 95°C -15 s, 65°C -60 s, 72°C -60 s; 72°C - 5 minutes
IBABP-L	ACATGGGTGA GCCGGAAAGG AGAC	CCGGAGTAGTGCTG GGACCAAGTGAAGT	95°C – 10 minutes 50 cycles: 95°C -15 s, 65°C -60 s, 72°C -60 s; 72°C - 5 minutes

2.5.4 Identification of Controls by Gene Expression Assays in Untreated Cells

Gene expression was determined in normal liver cDNA, normal intestinal cDNA, and untreated Huh7 cell line cDNA for each assay using the Taqman inventoried gene expression assays (Table 2.3) and in-house developed primers for PicoGreen gene expression assays (Table 2.4) above. Relative expression of each gene was determined by comparative C_T ($\Delta\Delta C_T$) method against the normal liver control values for each experimental condition (Bookout *et al.*, 2003). Figure 2.5 below outlines the representative expression of the genes assayed by Taqman inventoried gene expression assays against the β-2M housekeeping control gene in normal liver controls (n=1).

Figures 2.6 – 2.8 show the relative mean expression of FXR, SREBP-1c, ApoA1, PPARα, VLDLR, VDR, LXRα, ApoC-II, ApoC-III and HNF4α expressed in each of

the cell lines and normal intestine with relative expression compared to the normal liver controls. Figure 2.9 shows HSP70 relative mean expression against normal liver controls for each of the cell lines. Gene expression analysis was confirmed the target genes of interest were present in normal liver controls and Huh7 cell lines, however, the regulation of CYP7A1, IBABP-L and SLC10A1 were below the detection threshold of the assays used (in the cell lines) and further refinement of these assays was not undertaken as part of this research.

Huh7 was selected as the preferred cell line for gene expression analysis following evaluation of the results of the toxicity and untreated cell assays. This cell line selection was consistent with the requirements for a stable cell culture in antibiotic free media as discussed in Section 2.1 above.

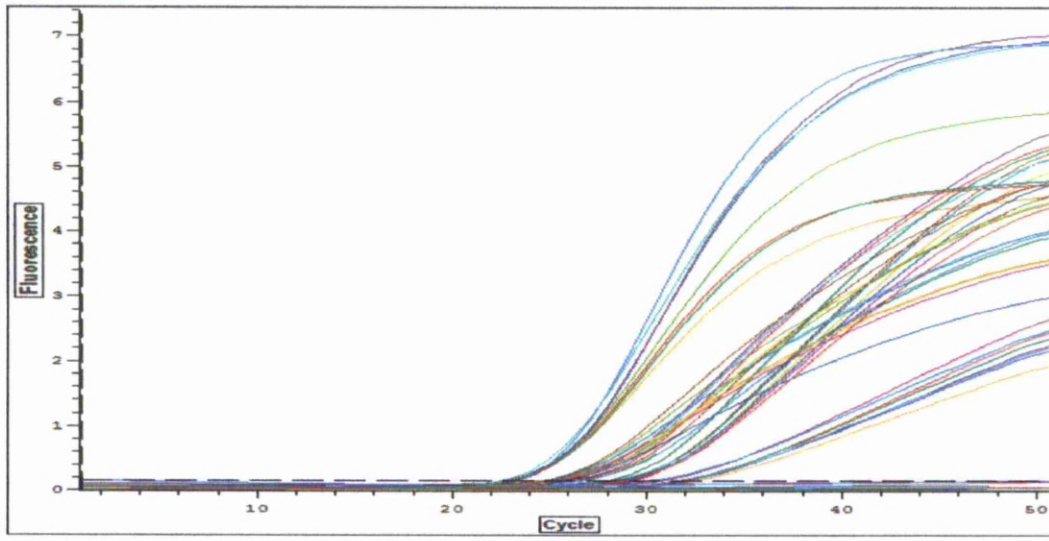


Figure 2.5: Opticon Trace: Representative Gene expression of FXR, SREBP-1c, ApoA1, PPARα, VLDLR, LXRα, ApoC-II, ApoC-III, SLC10A1, CYP7A1, GAPDH, and β-actin against β-2M housekeeping control in normal liver controls for Taqman gene expression assays, n=1

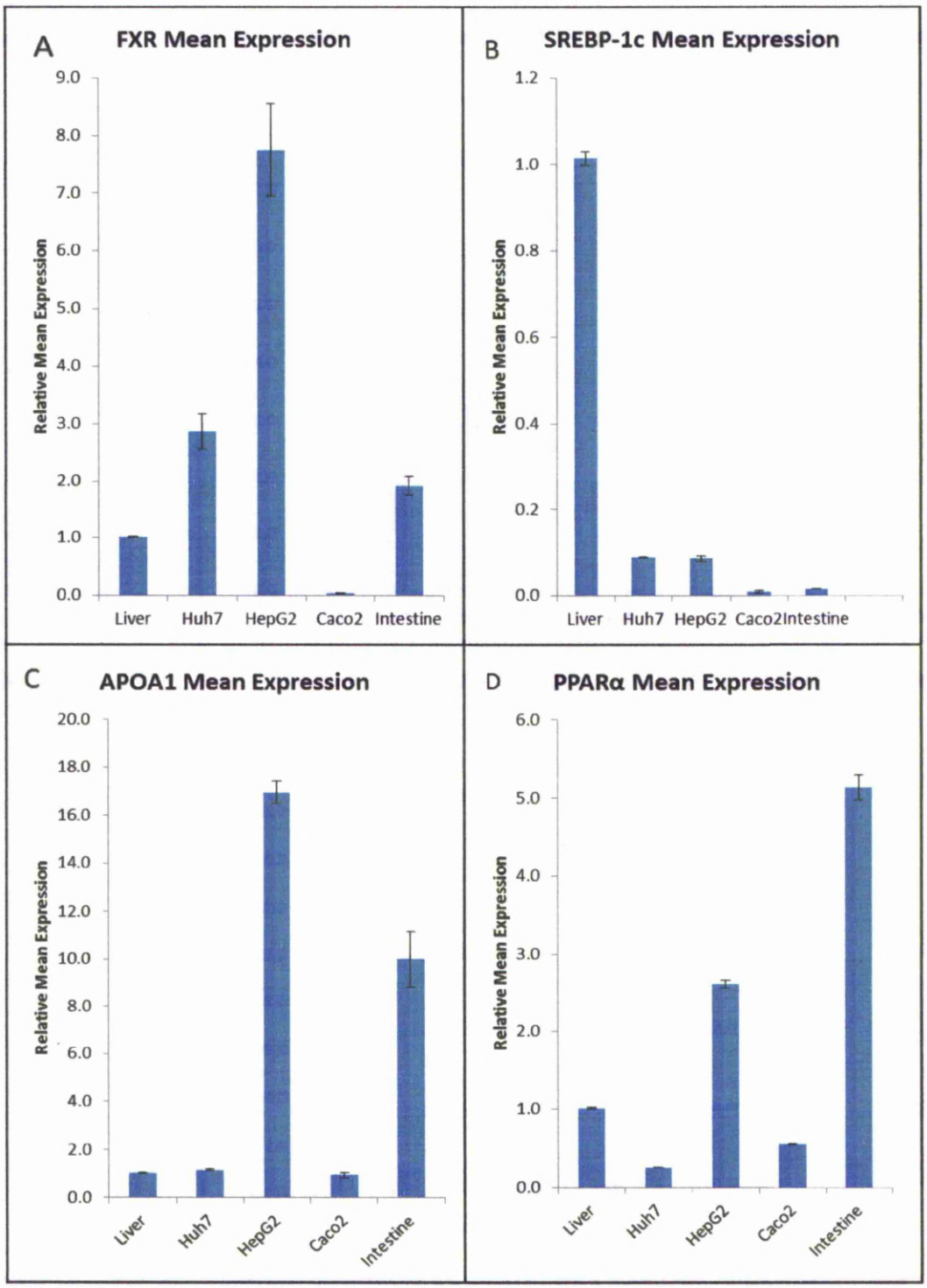


Figure 2.6: A= FXR Gene Expression Assay in untreated Huh7, HepG2 and Caco-2 cells with normal liver and normal intestinal controls; B = SREBP1-c Gene Expression Assay in untreated Huh7, HepG2 and Caco-2 cells with normal liver and normal intestinal controls; C = ApoA1 Gene Expression Assay in untreated Huh7, HepG2 and Caco-2 cells with normal liver and normal intestinal controls; D = PPARα Gene Expression Assay in untreated Huh7, HepG2 and Caco-2 cells with normal liver and normal intestinal controls; n=4 for all assays.

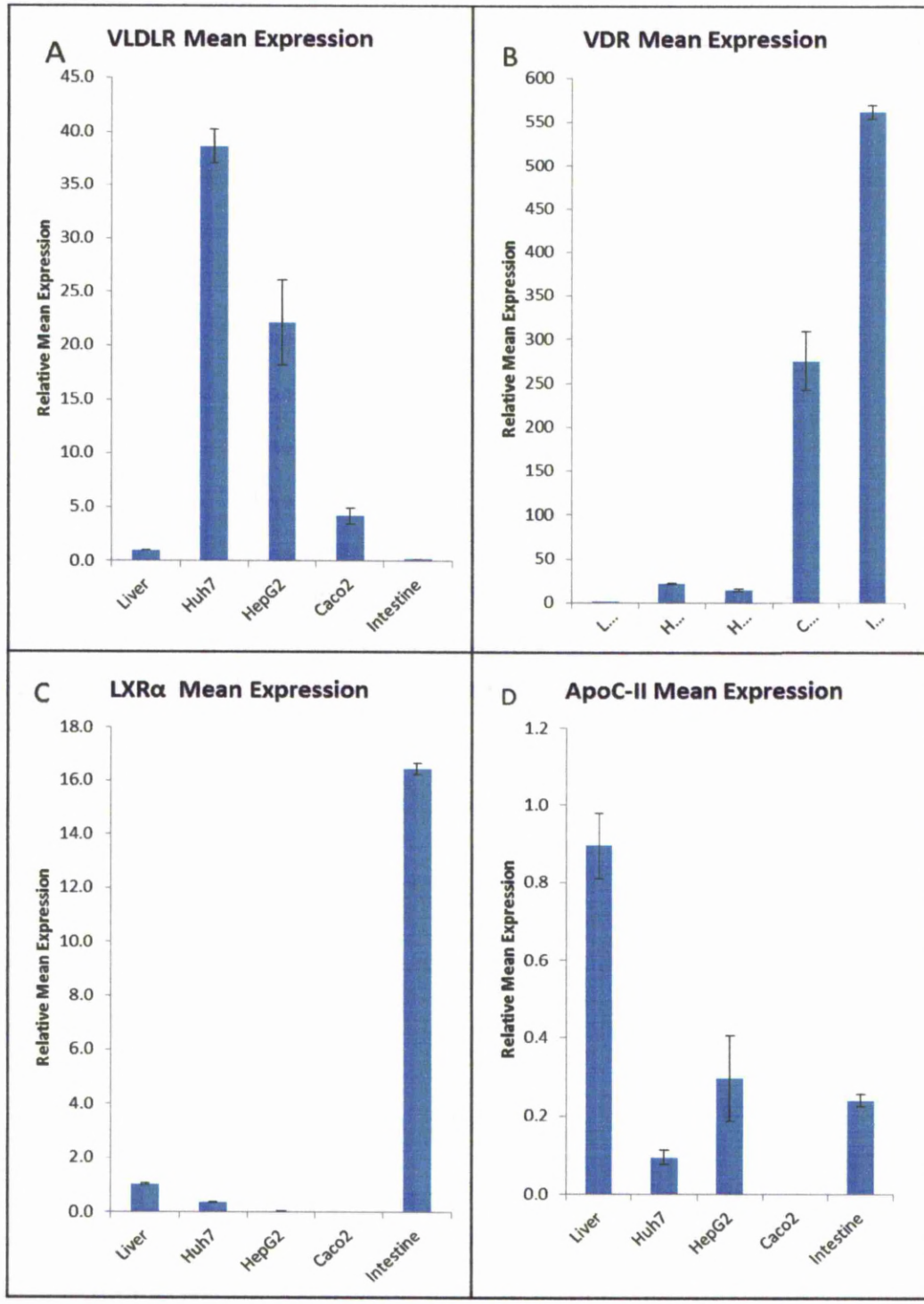


Figure 2.7: A = VLDLR Gene Expression Assay in untreated Huh7, HepG2 and Caco-2 cells with normal liver and normal intestinal controls; B = VDR Gene Expression Assay in untreated Huh7, HepG2 and Caco-2 cells with normal liver and normal intestinal controls; C = LXRα Gene Expression Assay in untreated Huh7, HepG2 and Caco-2 cells with normal liver and normal intestinal controls; and D = ApoC-II Gene Expression Assay in untreated Huh7, HepG2 and Caco-2 cells with normal liver and normal intestinal controls; n=4 for all assays.

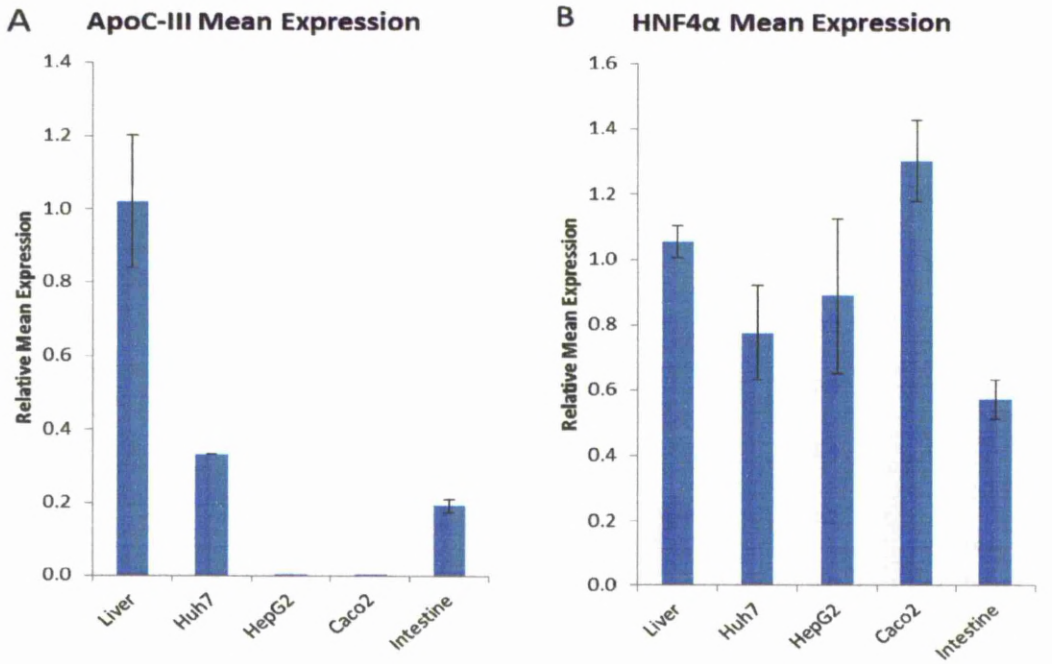


Figure 2.8: A = ApoC-III Gene Expression Assay in untreated Huh7, HepG2 and Caco-2 cells with normal liver and normal intestinal controls; and B = .HNF4α Gene Expression Assay in untreated Huh7, HepG2 and Caco-2 cells with normal liver and normal intestinal controls; for all assays.

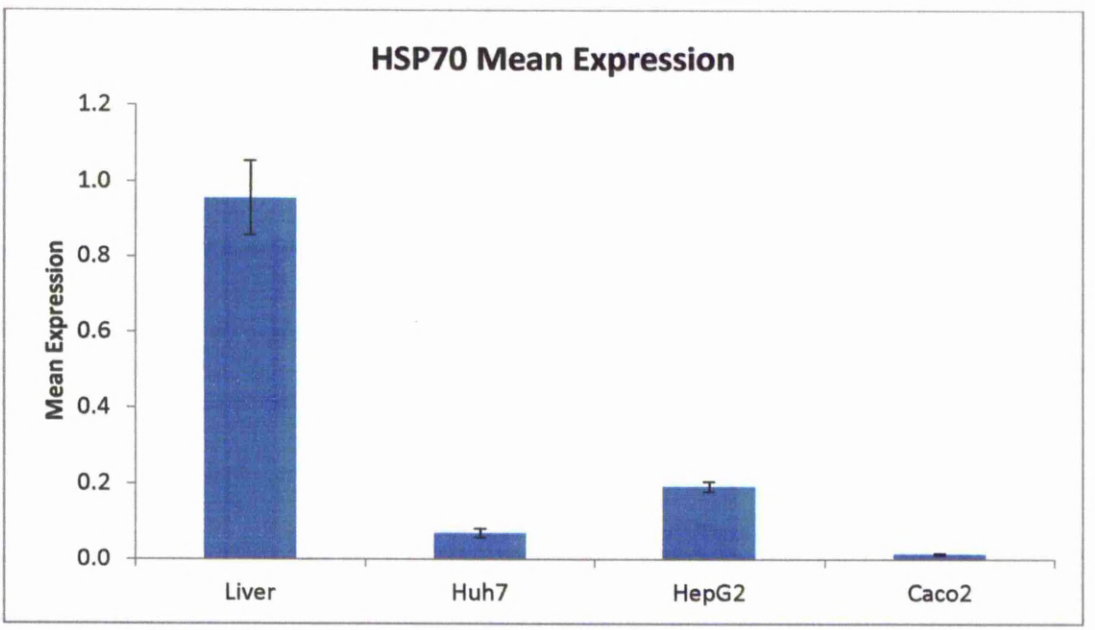


Figure 2.9: HSP70 Gene Expression Assay in untreated Huh7, HepG2 and Caco-2 cells with normal liver controls, n=4.

2.5.5 Cellular Accumulation of Ritonavir and Atazanavir

Cellular accumulation of RTV and ATV was determined as described above (2.3.2.6) in Huh7 cells at passage 14. The accumulation of ATV and RTV was determined at 0h and 24h post incubation with the drugs. Controls consisted of untreated Huh7 incubated in media. The results of the scintillation count were analysed to determine the cellular accumulation ratio (CAR) as described in Section 2.3.2.6.

The results showed that the cellular accumulation ratio (CAR) for ATV was 2 fold greater than it was for RTV (Figure 2.10, below).

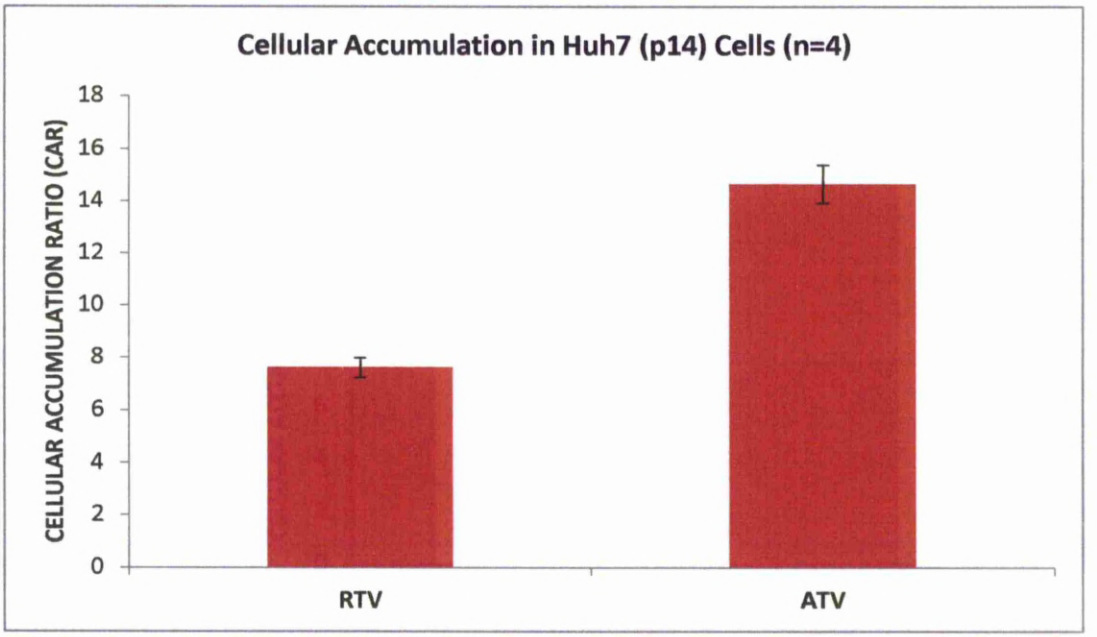


Figure 2.10: The comparative cellular accumulation (shown by cellular accumulation ratio (CAR)) of ritonavir (RTV) and atazanavir (ATV) in Huh7 cells (passage no. = 14), n=4.

2.5.6 Gene Expression in Ritonavir, Atazanavir and 22(S)-Hydroxycholesterol/ Ritonavir Treated Huh 7 Cells

Maximal induction and repression by both RTV and ATV was observed after 24h incubation. After 48h the induction and repression observed in the 24h RTV and ATV assays had diminished to return to basal expression levels. Consequently all assays were optimised for 24h incubation with drug treatments. The gene expression assay for FXR was evaluated for both the effects of RTV and ATV treatments, and to determine whether 22-(S)-hydroxycholesterol, an antagonist of LXRα, had regulated the FXR gene expression in the presence of RTV. As 22-(S)-hydroxycholesterol had been confirmed as not toxic in the range 0.01μM to 100μM (Section 2.5.2), a fixed concentration of 20μM was selected for the antagonist, as it had previously been shown to bind LXRα without activation at this concentration (Janowski *et al.*, 1999). HSP70 gene expression was stable across all concentrations of RTV, RTV plus 22-(S)-hydroxycholesterol and ATV in Huh7 cells (n=4) and was therefore selected as the negative control for the drug incubation experiments (Figure 2.11 below).

All results were quantified by calculating the relative mean expression of each gene and condition for each of the duplicates (n=4) against the relative mean expression of the housekeeping gene and plotted against the mean expression of each gene of the vehicle controls as previously described in Section 2.3.2.9.

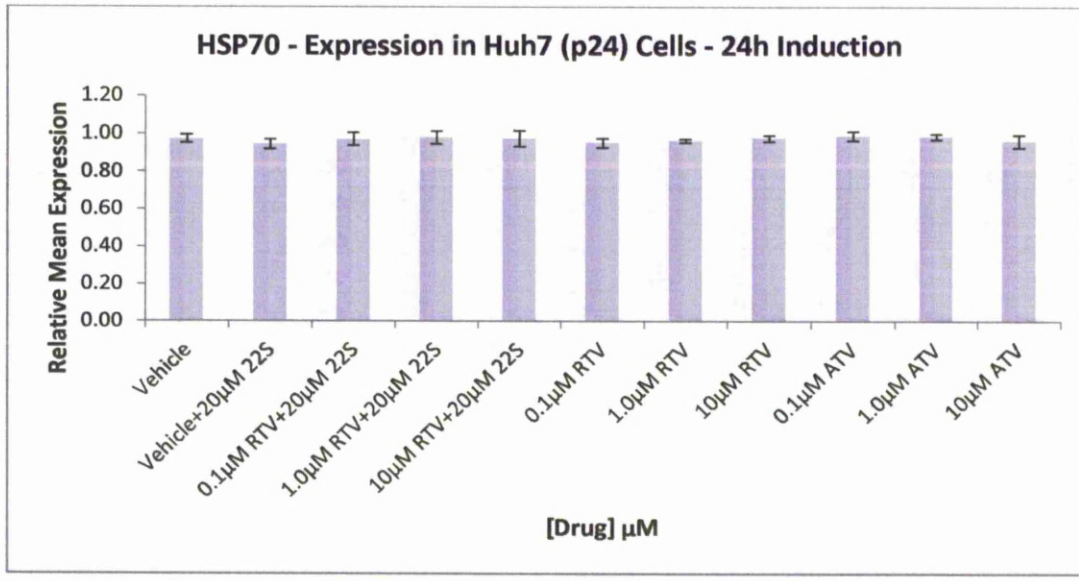


Figure 2.11: HSP70 Gene Expression Assay in Huh7 cells (passage no. = 24) incubated with ritonavir (RTV), RTV plus 20μM 22(S)-Hydroxycholesterol and atazanavir (ATV) for 24h, n=4.

Significant induction of ApoA-I by RTV was observed at concentrations of 0.1μM (2.7 fold increase compared to vehicle control, P=<0.0001), 1.0μM (3.6 fold increase compared to vehicle control, P=<0.0001) and 10μM (5.6 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.12 below. The statistically significant induction of ApoA-I by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM was abolished in the presence of 20μM 22-(S)-hydroxycholesterol, Figure 2.12 below. Significant induction of ApoA-I by ATV was also observed at a concentrations of 0.1μM (1.2 fold increase compared to vehicle control, P=0.0013), 1.0μM (1.4 fold increase compared to vehicle control, P=<0.0001) and 10μM (1.6 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), however, this was less marked than that which was observed for RTV, Figure 2.12 below.

ApoC-II was significantly induced by RTV at 0.1μM (1.7 fold increase compared to vehicle control, P=<0.0001), 1.0μM (3 fold increase compared to vehicle control,

P=<0.0001) and 10μM (9 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.12 below. The statistically significant induction of ApoC-II by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM was abolished in the presence of 20μM 22-(S)-hydroxycholesterol, Figure 2.12 below. ApoC-II was significantly repressed by ATV at concentrations of 0.1μM (4.4 fold decrease compared to vehicle control, P=<0.0001), 1.0μM (2 fold decrease compared to vehicle control, P=<0.0001) and 10μM (2.6 fold decrease compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.12 below.

Significant induction of ApoC-III at 0.1μM (1.2 fold increase compared to vehicle control, P=<0.0001), 1.0μM (1.3 fold increase compared to vehicle control, P=<0.0001) and 10μM RTV was observed (5 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.12 below. The use of 22-(S)-hydroxycholesterol abolished the significant induction of ApoC-III at 0.1μM, 1.0μM and 10μM RTV, Figure 2.12 below. ApoC-III gene expression was also significantly increased at 0.1μM (1.1 fold increase compared to vehicle control, P=0.0009), 1.0μM (1.1 fold increase compared to vehicle control, P=<0.0001) and 10μM ATV (1.1 fold increase compared to vehicle control, P=0.0022) in Huh7 cells (n=4), however, this was less marked than that which was observed for RTV, Figure 2.12 below.

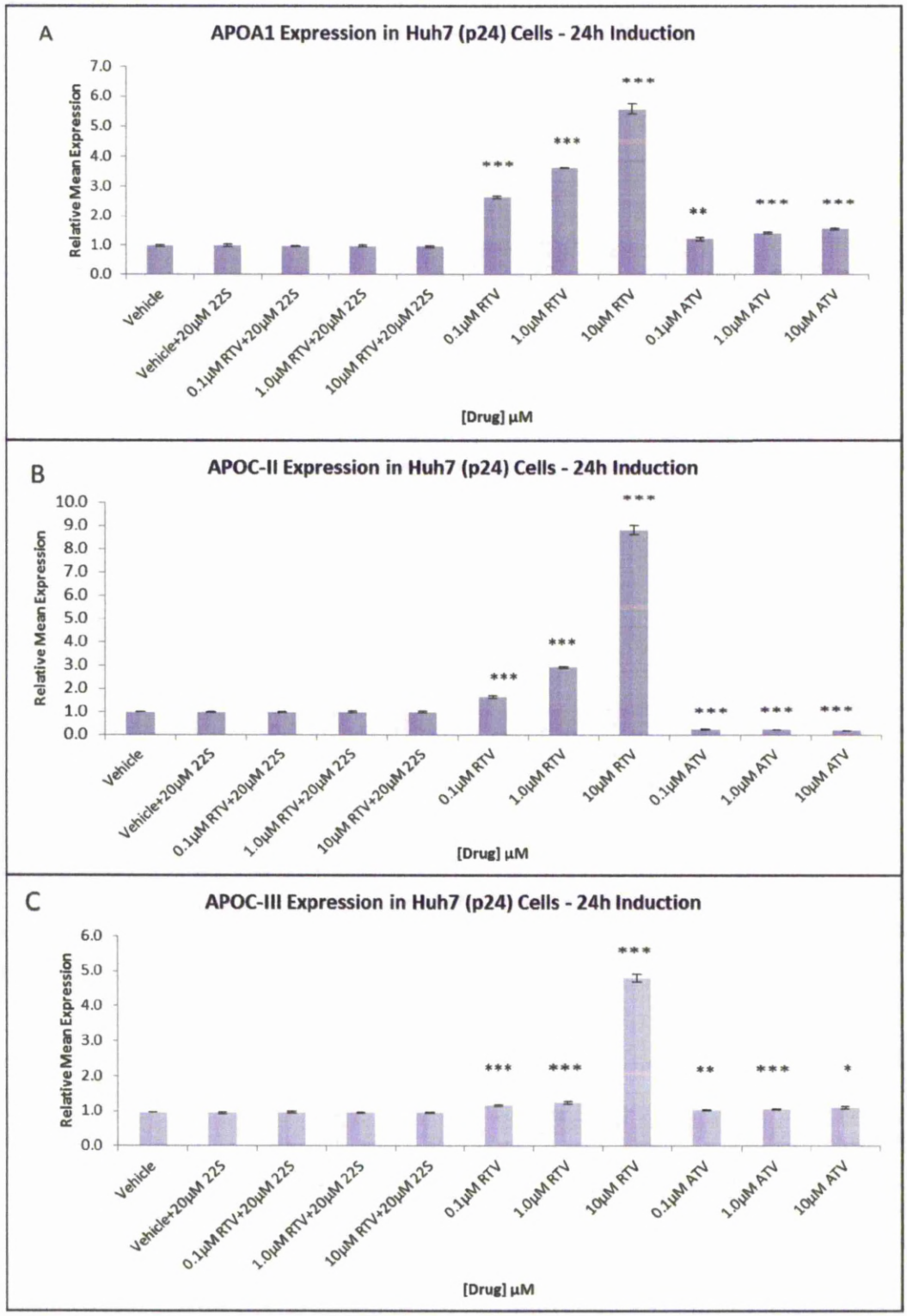


Figure 2.12: Gene expression assays in Huh7 cells (passage no. = 24) incubated with ritonavir (RTV), RTV plus 20μM 22(S)-Hydroxycholesterol and atazanavir (ATV) for 24h: A= ApoA-I; B = ApoC-II; and C = ApoC-III, n=4 for all assays. *** denotes P=<0.0001, ** denotes P=0.001* denotes P=<0.01.

PPARα gene expression was significantly repressed by RTV at concentrations of 0.1μM (2.6 fold decrease compared to vehicle control, P=<0.0001), 1.0μM (5.1 fold decrease compared to vehicle control, P=<0.0001) and 10μM (6.2 fold decrease compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.13 below. The repression of PPARα by RTV at concentrations of 0.1μM (2.6 fold decrease), 1.0μM (5.1 fold decrease) and 10μM (6.2 fold decrease) was abolished by the LXRα antagonist 22-(S)-hydroxycholesterol in Huh7 cells (n=4), Figure 2.13 below. PPARα gene expression was significantly down-regulated by ATV at the 10μM concentration only (1.1 fold decrease compared to vehicle control, P=<0.0001). At ATV concentrations of 0.1μM and 1.0μM there was no significant change in the expression of PPARα compared to the vehicle control, Figure 2.13 below.

Significant induction of SREBP-1c at 0.1μM (1.3 fold increase compared to vehicle control, P=<0.0001), 1.0μM (2.1 fold increase compared to vehicle control, P=<0.0001) and 10μM RTV was observed (3.2 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.13 below. The statistically significant induction of SREBP-1c at 0.1μM (1.3 fold increase), 1.0μM (2.1 fold increase) and 10μM (3.2 fold increase) observed after 24h treatment with RTV was abolished by the addition of 20μM 22-(S)-hydroxycholesterol at each of the RTV concentrations, Figure 2.13 below. Significant repression of SREBP-1c by ATV at concentrations of 0.1μM (1.3 fold decrease compared to vehicle control, P=<0.0001), 1.0μM (1.6 fold decrease compared to vehicle control, P=<0.0001) and 10μM (1.8 fold decrease compared to vehicle control, P=<0.0001) was observed after 24h induction in Huh7 cells (n=4), Figure 2.13 below.

VLDLR was significantly repressed by RTV at concentrations of 0.1μM (7.7 fold decrease compared to vehicle control, P=<0.0001), 1.0μM (15.8 fold decrease compared to vehicle control, P=<0.0001) and 10μM (10.9 fold decrease compared to vehicle control, P=<0.0001), in Huh7 cells (n=4), Figure 2.13 below. The significant repression of VLDLR by RTV at concentrations of 0.1μM (7.7 fold decrease), 1.0μM (15.8 fold decrease) and 10μM (10.9 fold decrease), was also abolished by the addition of 20μM 22-(S)-hydroxycholesterol at each RTV concentration, Figure 2.13 below. VLDLR was significantly induced by ATV at concentrations of 0.1μM (1.3 fold increase compared to vehicle control, P=0.0015), 1.0μM (1.6 fold increase compared to vehicle control, P=<0.0001) and 10μM (3.9 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.13 below.

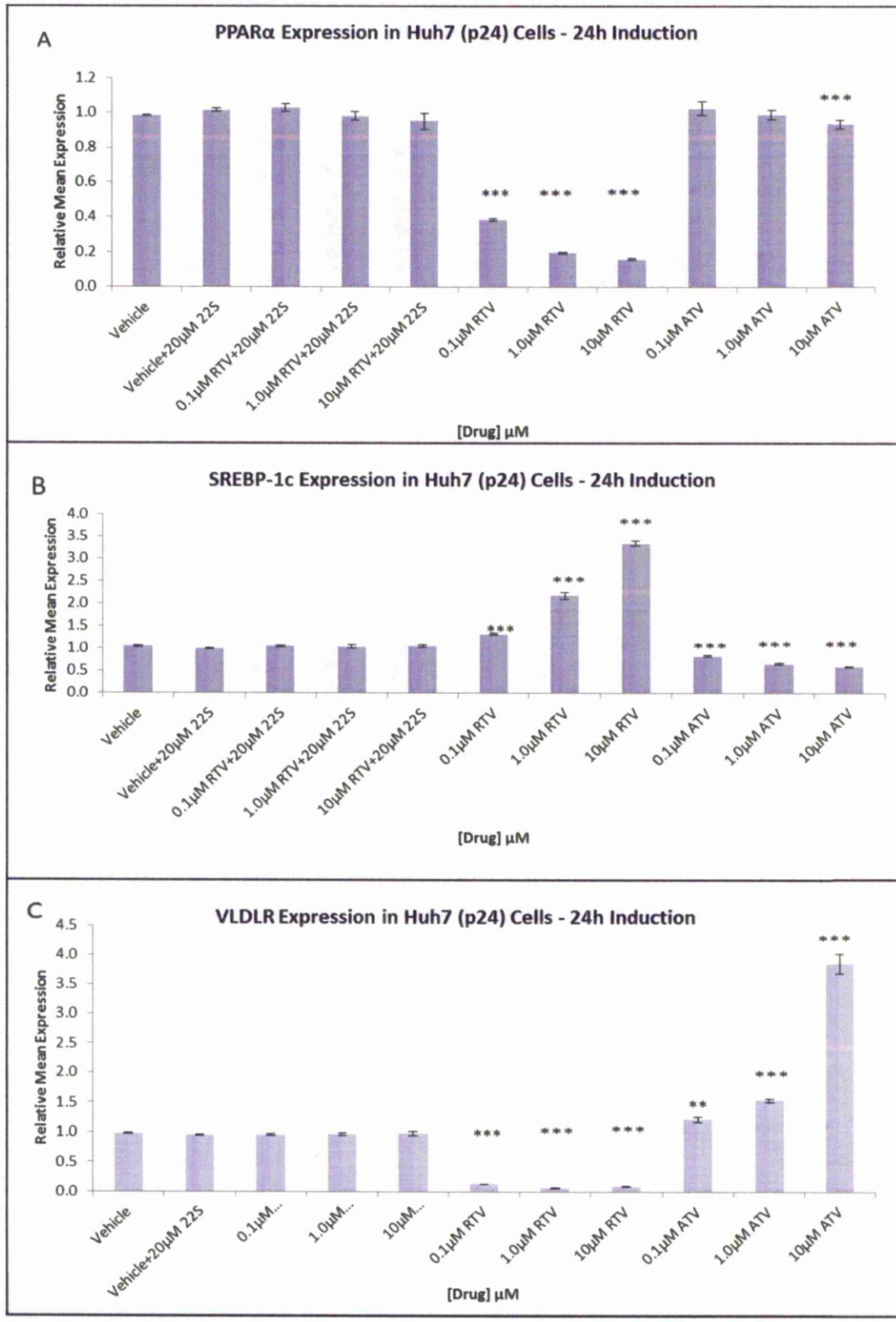


Figure 2.13: Gene expression assays in Huh7 cells (passage no. = 24) incubated with ritonavir (RTV), RTV plus 20μM 22(S)-Hydroxycholesterol and atazanavir (ATV) for 24h: A= PPARα; B = SREBP-1c; and C = VLDLR, n=4 for all assays. *** denotes P=<0.0001, ** denotes P=0.001, * denotes P=<0.01.

LXRα was also significantly induced by RTV concentrations of 0.1μM (3.8 fold increase compared to vehicle control, P=<0.0001), 1.0μM (6.7 fold increase compared to vehicle control, P=<0.0001) and 10μM (16.7 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.14 below. The addition of 20μM 22-(S)-hydroxycholesterol to each of the RTV concentrations abolished its significant induction of effects on LXRα in Huh7 cells (n=4), Figure 2.14 below. LXRα was significantly induced by ATV concentrations of 0.1μM (1.1 fold increase compared to vehicle control, P=0.042), 1.0μM (1.2 fold increase compared to vehicle control, P=0.0384) and 10μM (1.3 fold increase compared to vehicle control, P=0.0005) in Huh7 cells (n=4), however, this was less marked than that which was observed for RTV, Figure 2.14 below.

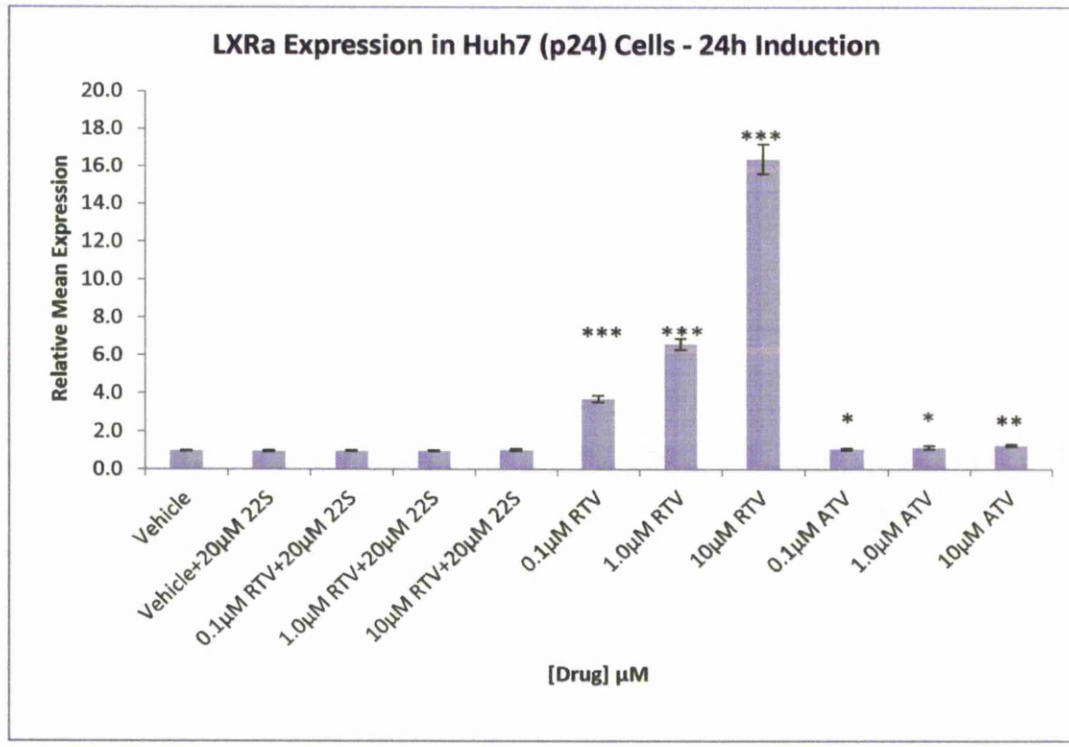


Figure 2.14: LXRα Gene Expression Assay in Huh7 cells (passage no. = 24) incubated with ritonavir (RTV), RTV plus 20μM 22(S)-Hydroxycholesterol and atazanavir (ATV) for 24h, n=4. *** denotes P=<0.0001, ** denotes P=<0.001, * denotes P=<0.05.

HNF4α was significantly repressed by RTV at concentrations of 0.1μM (1.4 fold decrease compared to vehicle control, P=<0.0001), 1.0μM (2.8 fold decrease compared to vehicle control, P=<0.0001) and 10μM (5.3 fold decrease compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.15 below. HNF4α, however, was also significantly repressed by RTV in the presence of 20μM 22-(S)-hydroxycholesterol at concentrations of 0.1μM (1.3 fold decrease compared to vehicle control, P=0.0035), 1.0μM (2.8 fold decrease compared to vehicle control, P=<0.0001) and 10μM (5.1 fold decrease compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.15 below. HNF4α was significantly induced by ATV at concentrations of 0.1μM (2.2 fold increase compared to vehicle control, P=0.0009), 1.0μM (2.5 fold increase compared to vehicle control, P=<0.0001) and 10μM (4 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.15 below.

FXR was significantly induced by RTV concentrations of 0.1μM (1.5 fold increase compared to vehicle control, P=<0.0001), 1.0μM (2.1 fold increase compared to vehicle control, P=<0.0001) and 10μM (1.5 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.15 below. FXR was also significantly induced by RTV in the presence of 20μM 22-(S)-hydroxycholesterol at concentrations of 0.1μM (1.4 fold increase compared to vehicle control, P=<0.0001), 1.0μM (2 fold increase compared to vehicle control, P=<0.0001) and 10μM (1.5 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.15 below. Significant repression of FXR gene expression was observed at ATV concentrations of 0.1μM (1.8 fold decrease compared to vehicle control, P=<0.0001),

1.0μM (1.8 fold decrease compared to vehicle control, P=<0.0001) and 10μM (2.1 fold decrease compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.15 below.

VDR was significantly induced by RTV concentrations of 0.1μM (1.5 fold increase compared to vehicle control, P=<0.0001), 1.0μM (2 fold increase compared to vehicle control, P=<0.0001) and 10μM (7.3 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.15 below. VDR was also significantly induced by RTV in the presence of 20μM 22-(S)-hydroxycholesterol at concentrations of 0.1μM (1.5 fold increase compared to vehicle control, P=<0.0001), 1.0μM (2.1 fold increase compared to vehicle control, P=<0.0001) and 10μM (7.6 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 2.15 below. VDR was significantly induced by ATV concentrations of 0.1μM (1.3 fold increase compared to vehicle control, P=0.0005), 1.0μM (1.6 fold increase compared to vehicle control, P=<0.0001) and 10μM (1.6 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), however, this was less marked than that which was observed for RTV, Figure 2.15 below.

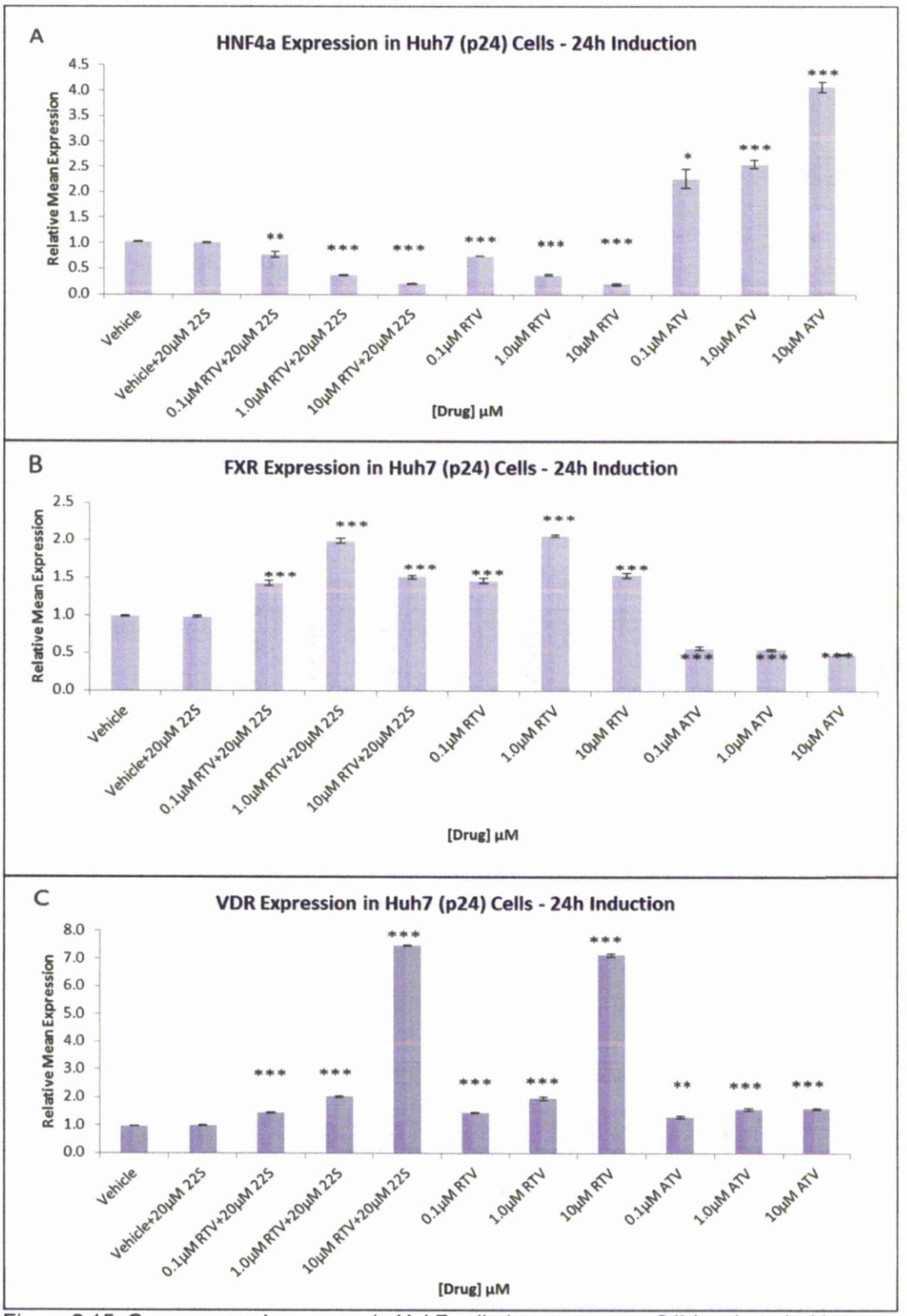


Figure 2.15: Gene expression assays in Huh7 cells (passage no. = 24) incubated with ritonavir (RTV), RTV plus 20μM 22(S)-Hydroxycholesterol and atazanavir (ATV) for 24h: A= HNF4α; B = FXR; and C = VDR, n=4 for all assays. *** denotes P=<0.0001, ** denotes P=0.001, * denotes P=<0.01.

2.6 Discussion

This Chapter investigated the specific impact of the PIs RTV and ATV on gene expression in NRs involved in lipid metabolism which were identified in Section 2.1 of this thesis, and on the gene expression of downstream targets in their metabolic pathways (Table 2.2). It considered whether the differential effects in gene expression in the presence of RTV and ATV could be attributed to differences in the cellular accumulation of these drugs. Finally it determined the effect of an LXRα antagonist (22-(S)-hydroxycholesterol) on gene expression in RTV and ATV treated Huh7 cells.

Cellular accumulation assays of RTV and ATV in the hepatic cell line, Huh7, indicated that the deleterious side effects associated with RTV were not due to higher intracellular accumulation of this drug. The results (Section 2.5.5) demonstrated that ATV intracellular accumulation was 2 fold greater than it was for RTV. It was concluded, therefore, that intracellular accumulation was not influencing the contrasting metabolic profiles of these drugs.

Gene expression analysis was conducted using Taqman Gene Expression inventoried assays for RT-PCR (Applied Biosystems Limited, Warrington, UK) and in-house developed real time PCR gene expression assays for quantification by Pico-green. The Taqman Gene Expression inventoried assays for RT-PCR (Table 2.3) were used for FXR, LXRα, ApoA-I, ApoC-II, ApoC-III, SREBP-1C, VLDLR, SLC10A1, PPARα, CYP7A1, GAPDH and β-actin. The in-house developed real time PCR gene expression assays for quantification by Pico-green were used to determine the gene expression of HSP70, IBABP-L, VDR and HNF4α. Development and optimisation

of these assays was as described in Sections 2.3.2.7 and 2.5.3. All gene expression was quantified against the housekeeping gene, β-2-microglobulin (β-2M), a Taqman inventoried Gene Expression assay.

FXR activation improves serum lipid profiles through suppression of hepatic triglyceride synthesis via repression of SREBP-1c and increased expression of lipid metabolism genes including PLTP, LCAT and ApoC-II (Fiorucci *et al.*, 2007). The observed significant up-regulation of ApoC-II (P=<0.0001 at 0.1μM, 1μM and 10μM) and significant down-regulation of HNF4α (P=<0.0001 at 0.1μM, 1μM and 10μM) gene expression was consistent with the expected outcome of the significant induction of FXR (P=<0.0001 at 0.1μM, 1μM and 10μM) by RTV. The addition of the LXRα antagonist 22(S)-hydroxycholesterol to the RTV induction experiments did not knock-out the up-regulation of FXR or the down-regulation of HNF4α by RTV. The up-regulation of ApoC-II by RTV was, however, completely abolished in the presence of the LXRα antagonist suggesting that more than one mechanism is involved in its up-regulation. FXR was significantly down-regulated in response to ATV induction studies (P=<0.0001 at 0.1μM, 1μM and 10μM) whilst HNF4α gene expression was significantly up-regulated by ATV (P=0.0009 at 0.1μM, and P=<0.0001 at 1μM and 10μM) suggesting that down-regulation of FXR may be the mechanism involved with this response. This was not investigated further in this thesis as ATV has a better lipid profile than RTV and the focus of this research was directed towards the metabolic target genes where the observed effect was inconsistent with regulation by FXR. ApoC-II, a mediator of TG metabolism, was significantly down-regulated by all concentrations of ATV induction ((P=<0.0001 at

0.1μM, 1μM and 10μM with a maximal 4.4 fold decrease compared to vehicle control at 0.1μM), which may be attributed to regulation by PPARα. Again this was not further investigated during this research due to the contrasting lipid profile of ATV which is better than that of RTV, with the focus remaining on the deleterious metabolic side effects of the latter.

SREBP-1c is a target gene of FXR via a SHP-1 dependent pathway. The consequence of FXR down-regulation of SREBP-1c *in vivo* would be the repression of hepatic triglyceride and fatty acid synthesis (Zhang *et al.*, 2008). Although FXR mediated inhibition of fatty acid synthesis may be transient. Prolonged exposure to high levels of bile acids or potent agonists does not appear to have the same beneficial effects as short term exposure on plasma lipids. SHP-1 mediated suppression of SREBP-1c via FXR activation appears to be overcome through the direct activation of fatty acid synthase (FAS) by FXR which is necessary for sufficient fatty acid synthesis under states of cholesterol and bile acid excess to avoid cholesterol toxicity (Fiorucci *et al.*, 2007). The up-regulation of FXR by RTV should therefore have resulted in the inhibition of SREBP-1c via SHP-1, if this pathway was dominating the regulation of this gene.

The RTV induction assays resulted in a significant up-regulation of SREBP-1c ($P=<0.0001$ at 0.1μM, 1μM and 10μM). When this is compared to a concentration dependent significant increase in FXR expression, it suggests another mechanism was involved with SREBP-1c up-regulation. There is evidence that the effects of LXRα on fatty acid synthesis are mediated through the increased expression of

SREBP-1c, and that the latter is a transcription factor which has been associated with PI induced lipodystrophy (Telenti *et al.*, 2002). As activated LXRα stimulates lipogenesis by inducing SREBP-1c and other lipogenic genes (Handschin *et al.*, 2005), and significant up-regulation of LXRα was observed at all RTV concentrations (P=<0.0001 at 0.1μM, 1μM and 10μM, with maximal 16.7 fold up-regulation at 10μM RTV), it is likely that LXRα mediated the up-regulation of SREBP-1c in these assays. LXRα chemical antagonism using 22(S)-hydroxycholesterol abolished the induction effects of RTV on both LXRα and SREBP-1c supporting this hypothesis. Furthermore SREBP-1c gene expression was significantly down-regulated at all concentrations of ATV (P=<0.0001 at 0.1μM, 1μM and 10μM) suggesting a potential explanation for the contrasting lipid profiles of ATV and RTV.

LXRα has been shown to inhibit PPARα signalling (Miyata *et al.*, 1996). PPARα was significantly down-regulated at all concentrations (P=<0.0001 at 0.1μM, 1μM and 10μM) in the RTV assays. LXRα inhibition with 22(S)-hydroxycholesterol also abolished the effects of RTV on PPARα supporting the hypothesis that LXRα is a key player in the regulation of PPARα. At 0.1μM and 1μM ATV there was no significant change in PPARα gene expression from vehicle controls. The significant down-regulation of PPARα by ATV observed at 10μM (maximal decrease of 1.1 fold compared to vehicle, P=<0.0001) was to a lesser magnitude than observed in the RTV experiments (maximal decrease of 6.2 fold compared to vehicle observed at 10μM RTV). This may reflect the magnitude of difference between the increase in LXRα expression by ATV (maximal increase of 1.3 fold compared to vehicle

observed at 10μM, P=0.0005) compared to the increase in LXRα expression by RTV (maximal increase of 16.7 fold compared to vehicle observed at 10μM, P=<0.0001).

The induction of ABC transporters by LXRα increases the transfer of cholesterol and phospholipids to ApoA-I containing lipoproteins (Kalaany *et al.*, 2006; Pascussi *et al.*, 2008). The observed significant induction of VDR, like FXR, by RTV should result in repression of ApoA-I activity, but significant induction was observed at all RTV concentrations (P=<0.0001 at 0.1μM, 1μM and 10μM). This supports the theory that up-regulation of LXRα target genes may be the mechanism involved with the significant up-regulation of ApoA-I, supported by the abolition of this up-regulation when the LXRα antagonist, 22(S)-hydroxycholesterol was co-incubated with RTV. The significant up-regulation of ApoA-I by ATV (maximal increase of 1.6 fold compared to vehicle observed at 10μM ATV, P=<0.0001) was to a much lesser magnitude than observed in the RTV experiments (maximal increase of 5.6 fold compared to vehicle observed at 10μM RTV, P=<0.0001). This also may be attributed to the magnitude of difference between the increase in LXRα expression by ATV (maximal increase of 1.3 fold compared to vehicle observed at 10μM) compared to the increase in LXRα expression by RTV (maximal increase of 16.7 fold compared to vehicle observed at 10μM).

PPARα induction leads to the repression of ApoC-III (Duval *et al.*, 2007; Feldman *et al.*, 2008; Yamada *et al.*, 2007). In the RTV assays, the significant down-regulation of PPARα may have contributed to the significant induction of ApoC-III, (a 5 fold increase compared to the vehicle control at 10μM RTV, P=<0.0001), as LXRα

induces ApoC-III through its repression of PPARα which was significantly down-regulated at all RTV concentrations. Furthermore the inhibition of LXRα by 22(S)-hydroxycholesterol abolished the effects of RTV on ApoC-III (and PPARα) supporting the hypothesis that LXRα is a key player in the regulation of both ApoC-III and PPARα. ApoC-III gene expression was significantly up-regulated by a magnitude of 1.1 fold at all ATV concentrations (P=0.0009 at 0.1μM, P=<0.0001 at 1.0μM and P=0.0022 at 10μM) which may be a contributory factor in the mechanisms involved with the contrasting lipid profiles of ATV and RTV.

LXRα also represses VLDLR (Scotti *et al.*) which was significantly down-regulated at all RTV concentrations (P=<0.0001 at 0.1μM, 1μM and 10μM) with the maximal 15.8 fold decrease compared to vehicle seen at 1.0μM RTV. Inhibition of LXRα with 22(S)-hydroxycholesterol abolished the effects of RTV on VLDLR supporting the hypothesis that LXRα was regulating VLDLR. The statistically significant up-regulation of VLDLR by all concentrations of ATV (P=0.0015 at 0.1μM, and P=<0.0001 at 1μM and 10μM, with the maximal increase of 3.9 fold compared to vehicle observed at 10μM) is consistent with the regulation of this gene by FXR and by PPARα regulation. This may again underpin the different mechanisms involved in the contrasting lipid profiles of ATV and RTV.

The use of RTV at low doses in combination with other PIs exploits its role as a pharmacoenhancer to inhibit their metabolism (Foisy *et al.*, 2008). RTV is no longer used at therapeutic doses due to its high toxicity and the availability of other PIs with more attractive lipid profiles (Foisy *et al.*, 2008) including ATV (Barreiro *et al.*,

2005). The beneficial effects of RTV as a pharmacoenhancer also make it a notorious mediator of drug-drug interactions and toxological effects due to non-clearance of these drugs (Barreiro *et al.*, 2005; Foisy *et al.*, 2008). PIs boosted by RTV still carry a high pill burden as part of twice daily regimens and the probability of treatment interruption due to drug induced toxicity is 2 fold greater with RTV than with ATV. Furthermore patients taking a RTV boosted regimen have incurred significant increases in total cholesterol and triglyceride levels after 48 week trials whereas the same parameter in the ATV cohort were unchanged (Barreiro *et al.*, 2005). The differences in the cellular accumulation of RTV and ATV shown in Section 2.5.5 are important as it proves the hypothesis that the differential metabolic effects observed in the RTV assays were not due to higher intracellular accumulation and supports the hypothesis that these drugs target different genes in the lipid metabolism complex.

Current research continues to examine the role and impact of individual and multiple NR "knockdown" on the induction profile of the target genes of the metabolic NRs, thereby determining the relative contribution of FXR, LXRα and PPARα in both a drug-specific and tissue-specific manner. Chapter 3 of this thesis will discuss the comparative effects of biological knockout of LXRα on the differing gene expression profiles associated with RTV and ATV in the Huh7 cell line, in an effort to confirm the specific influence of LXRα in these findings.

CHAPTER 3

THE INFLUENCE OF SIRNA KNOCKDOWN OF LIVER OXYSTEROL RECEPTOR ALPHA (LXRα) IN RITONAVIR TREATED HEPATIC CELLS

3.1 Introduction

Chapter 2 of this thesis outlined the role of the metabolic NRs together with the role of the PIs RTV and ATV as substrates for/activators of these NRs (Dussault *et al.*, 2001; Healan-Greenberg *et al.*, 2008; Lehmann *et al.*, 1998). Chapter 2 also discussed the impact that PIs have been shown to have on several molecular pathways key to lipid metabolism including intra-nuclear transcription factors and the nuclear proteasome (Lagathu *et al.*, 2005; Mallon, 2007). The regulatory mechanisms and target genes for the metabolic NRs have been outlined in Chapter 1 – General Introduction of this thesis and the complex nature of the metabolic NRs involved in bile acid metabolism and detoxification together with genes and transporters regulated by the activation of the NRs is outlined in Figure 2.1, (Chapter 2). In Chapter 2 the role of FXR and LXRα as master regulators of cholesterol homeostasis, and modulators of the transcription of many key genes involved in drug and lipid metabolism including SREBP-1c, a key player in lipogenesis (Hui, 2003; Liang *et al.*, 2001; Osborne, 2000) was discussed. This Chapter continues to investigate the role of the metabolic NRs in regulating lipid and bile homeostasis in response to PI metabolism.

In Chapter 2, the LXRα antagonist 22-(S)-hydroxycholesterol was used to chemically inhibit LXRα gene expression. An antagonist reduces the effects of an agonist, which may be an endogenous ligand or a drug, by either competing for the receptor binding site to stop the agonist binding thereby reducing any potential agonist effects. This may be reversible or irreversible binding, the former being concentration dependent and the latter due to covalent bind to the receptor. Other

antagonists may be non-competitive (binding an alternate site to block the agonist binding the receptor) or functional, exerting the opposite effect to the agonist, by binding an alternate site and through which it actions as an agonist, and reducing the effect of the agonist at the first receptor. 22-(S)-hydroxycholesterol has previously been shown to bind LXRα without activation (Janowski *et al.*, 1999) and was therefore considered a suitable LXRα antagonist to determine the involvement of LXRα in the metabolic NR response to RTV. The use of a chemical antagonist to validate the effects of RTV on LXRα gene expression and its regulation of target gene expression has limitations even for the best characterised ligands as it is unwise to ignore the possibility that RTV and/or the antagonist may be influencing this gene expression through promiscuity at more than one target.

Biological knockdown of a gene by siRNA causes a transient knockdown of gene expression by degradation of the mRNA transcript responsible for protein synthesis (Dykxhoorn *et al.*, 2006). The effects are transient because new protein can be synthesised when new mRNA is transcribed. The duration of the knockdown will vary between genes so it is necessary to validate the knockdown at both the gene expression and protein levels.

Chapter 2 also discussed the observed impact of RTV up-regulation of FXR gene expression on its target genes ApoC-II (significantly up-regulated) and HNF4α (significantly down-regulated) It contrasted these findings with the reported role of FXR activation in the improvement of serum lipid profiles through repression of SREBP-1c and increased expression of PLTP, LCAT and ApoC-II (Fiorucci *et al.*,

2007). RTV effects on HNF4α were not influenced by co-incubation with the LXRα antagonist 22(S)-hydroxycholesterol, supporting the hypothesis of FXR influencing its regulation. The up-regulation of ApoC-II by RTV was, however, completely abolished in the presence of the LXRα antagonist, supporting the hypothesis that both FXR and LXRα influence the regulation of ApoC-II. This chapter further investigates the influence of LXRα inhibition on ApoC-II gene expression in the presence of RTV.

In Chapter 2, it was hypothesised that LXRα had mediated the up-regulation of SREBP-1c, as the LXRα antagonist 22(S)-hydroxycholesterol abolished the induction effects of RTV on both LXRα and SREBP-1c. In this Chapter, knockdown of LXRα by small interfering RNA (siRNA) is used to probe the findings of Chapter 2 and to further assess the influence of RTV on the previously identified target genes of LXRα and FXR. It was also hypothesised that LXRα inhibits PPARα gene expression as this was significantly down-regulated in the RTV assays and these effects were abolished in when RTV was co-incubated with the LXRα antagonist 22(S)-hydroxycholesterol. The use of siRNA knockdown of LXRα will seek to validate this.

It has been reported that PPARs may regulate metabolic gene expression by the formation of a NR heterodimer with LXRα (Giguere, 1999). PPARα induction leads to the repression of ApoC-III (Duval *et al.*, 2007; Feldman *et al.*, 2008; Yamada *et al.*, 2007). In the RTV assays, Chapter 2, the observed significant down-regulation of PPARα may have influenced the significant up-regulation of ApoC-III, as LXRα

induces ApoC-III through its repression of PPARα. Co-incubation with LXRα antagonist, 22(S)-hydroxycholesterol, abolished the effects of RTV on ApoC-III (and PPARα) supporting the hypothesis that LXRα is involved in the regulation of both these genes. It is further hypothesised VLDLR gene expression which was significantly down-regulated at all RTV concentrations was inhibited via LXRα up-regulation as co-incubation with LXRα antagonist 22(S)-hydroxycholesterol abolished the effects of RTV on VLDLR.

The observed significant induction of VDR, like FXR, by RTV in Chapter 2 should have resulted in the down-regulation of ApoA-I expression, but the opposite effect was observed at all RTV concentrations. It is hypothesised that the up-regulation of LXRα target genes may be influencing the significant up-regulation of ApoA-I, a theory supported by the abolition of this up-regulation when the LXRα antagonist, 22(S)-hydroxycholesterol was co-administered with RTV.

Current research continues to examine the role and impact of individual and multiple NRs on the induction profile of the target genes, thereby determining the relative contribution of FXR, LXRα and PPARα in both a drug-specific and tissue-specific manner. This Chapter will discuss the comparative effects of biological knockout of LXRα by siRNA on the gene expression profiles associated with RTV in the Huh7 cell line, in an effort to confirm the specific influence of LXRα in these findings.

3.2 Aim

The aims of this research were to further investigate the specific impact of the PI RTV on gene expression in NRs involved in lipid metabolism identified in Section 2.1 of this thesis, and on the gene expression of downstream targets in their metabolic pathways (Table 2.2); to confirm the differential effects in gene expression in the presence of RTV and RTV plus 22(S)-hydroxycholesterol seen in Section 2.5.6 and to establish the effect of using siRNA knockdown of LXRα on gene expression in RTV treated cells.

3.3 Materials and Methods

3.3.1 Materials

As previously described in Section 2.3.1 above. CelLytic M and Protease Inhibitor Cocktail were purchased from Sigma Aldrich Co. Limited (Irvine, Ayrshire, UK). Lipofectamine 2000 Transfection Reagent and Novex Western Protein Kit including NuPage Precast Gels and iBlot kits were purchased from Invitrogen Limited (Paisley, UK). LXRα, GAPDH and non-Targeting small interfering RNAs (siRNAs) were purchased from Dharmacon-Thermo Scientific (Epsom, UK). Monoclonal mouse anti-human antibody for GAPDH was purchased from Abcam PLC (Cambridge, UK). Monoclonal mouse anti-human antibodies for LXRα and β-actin, and goat anti mouse secondary antibody were purchased from R and D Systems Europe Limited (Abingdon, Oxon, UK).

3.3.2 Cells and Cell Maintenance

3.3.2.1 Huh7 Cell Line

As previously described in Section 2.3.2.1 above.

3.3.2.2 MTT Cell Toxicity Assays

As previously described in Section 2.3.2.2 of this thesis.

3.3.2.3 Cell Treatment

The Huh7 cell line was seeded at a density of $2.5x10^6$ per well into 10% FCS-supplemented DMEM medium, in Nunclon Up-Cell Surface 6 well plates and incubated at 37°C and 5% CO_2 for 24h prior to the set-up of all biological knockdown experiments. The concentration–response experiments for RTV, ATV and analysis of methanol treated controls were conducted as previously described in Section 2.3.2.3 above. The concentration–response experiments for RTV in the biological knockdown treated Huh7 cells were conducted at final concentrations of 0, 0.1, 1.0, and 10μM RTV following an initial 24h knockdown by siRNA. The cells were incubated at 37°C and 5% CO_2 and were sampled at 24h.

3.3.2.4 RNA Isolation from Huh7 Cells

Isolation of RNA from untreated, RTV-treated, ATV-treated and siRNA treated Huh7 cells was undertaken using the Tri®Reagent method as described in 2.3.2.4.

3.3.2.5 Production of cDNA

The cDNA was synthesised as previously described in Section 2.3.2.5 above.

3.3.2.6 Identification of Preferred Housekeeping Control by Gene Expression Assay

Selection of suitable candidate housekeeping controls was determined by gene expression analysis in Huh7 cells (n=3) in three conditions: untreated cells, mock transfections (Lipofectamine 2000 vehicle with siRNA vehicle only) and siRNA knockdown of LXRα using Taqman Endogenous Control Plates. The results of the

initial analysis revealed five potential candidates: Glyceraldehyde Phosphate Dehydrogenase (GAPDH); β-actin; Hydroxymethyl-bilane Synthase (HMBS); and 60S Acidic Ribosomal Protein (also known as human Large Ribosomal Protein, RPLPO) Figure 3.1, below; and β-2-microglobulin (β-2M, Figure 3.2). Large volume endogenous control assays were not available for all individual Taqman inventoried assays so further analysis was performed on β-actin and β-2M across all treatments in Huh7 cells.

3.3.2.7 Optimisation of LXRα Knockdown in the Huh7 Cell Line

LXRα was identified as a target for biological knockdown to facilitate further investigation of its regulation of gene expression in response to 24h exposure to RTV. GAPDH was identified as a suitable positive control for siRNA knockdown as it had previously been determined to have stable gene expression levels 24h exposure to RTV and RTV plus 20μM 22-(S)-hydroxycholesterol, and in untreated Huh7 cells. The negative control of Dharmacon Non-Targeting siRNA was selected on the basis of previous experience within the Liverpool HIV Pharmacology Group (LHPG).

Lipofectamine 2000 (Invitrogen, UK) was used to transfect Huh7 cells using a matrix of Lipofectamine 2000 (0.5μl, 1.0μl and 1.5μl) and siRNA concentrations (0nM, 5nM, 10nM, 15nM and 20nM) in 48 well plates to optimise the conditions for maximal knockdown at the mRNA level over 3 time periods: 24h, 48h and 72h in accordance with the manufacturer's instructions. Each reaction was set up with 4 biological and 4 technical replicates. Controls were set up for each time period as untreated cells and vehicle controls with 0.5μl, 1.0μl and 1.5μl Lipofectamine plus 25μl of 1 x siRNA buffer (prepared in accordance with Dharmacon instructions) per

reaction. Transfections were performed in accordance with the manufacturer's standard protocol for Lipofectamine 2000 (Invitrogen, UK). Briefly, the day prior to transfection $2x10^5$ Huh7 (at passage 22) cells were plated in 250µl 10% FCS/DMEM culture medium so that at transfection they would be 30-50% confluent. The culture media was replaced by pre-warmed (37°C) serum free DMEM media on the day of transfection. LXRα, GAPDH (positive control) and non-targeting (negative control) siRNAs were diluted in 1x siRNA buffer (25µl) (prepared in accordance with Dharmacon instructions) to provide concentrations to give final reaction concentrations in the range 5nM to 20nM, and incubated for 5 minutes at room temperature whilst the diluted Lipofectamine 2000 in serum free DMEM (25µl) media was prepared. Following incubation the dilute siRNA and dilute Lipofectamine 2000 were combined and incubated at room temperature for a further 20 minutes. The siRNA/Lipofectamine 2000 complexes were added to the appropriate wells containing cells and were mixed gently by rocking the plate. Each well volume was made up to 200µl by the addition of a further 150µl serum free DMEM media. The plates were incubated at 37°C/5% CO_2 for 4 hours. Following 4 hour incubation the siRNA/Lipofectamine 2000 complex containing media was completely aspirated and replaced by pre-warmed (37°C) 10% FCS/DMEM media prior to further incubation at 37°C/5% CO_2 for 24h, 48h or 72h, until ready for analysis of gene knockdown efficiency by RT-PCR.

Following optimisation confirmation of the preferred conditions for maximum efficiency mRNA knockdown, the transfections were repeated scaled-up in accordance with the manufacturer's instructions to facilitate optimisation of protein

knockdown. Briefly, Lipofectamine 2000 was used to transfect $2.5x10^6$ Huh7 (at passage 23) cells plated in 2.5ml (final volume) media using 5µl Lipofectamine 2000 and LXRα siRNA (15nM), GAPDH (20nM) and non-targeting siRNA (20nM) final concentrations in 6 well Nunclon Up-Cell plates to optimise the conditions for maximal knockdown at the protein and mRNA levels over 3 time periods: 24h, 48h and 72h as described above. Nunclon Up-Cell 6 well plates (for temperature sensitive release of adherent cells) were used for scaled up assays as they facilitated protein and RNA extraction from the same lysate. Efficiency of the siRNA knockdown was determined by RT-PCR.

Protein was extracted using CelLytic M and protease inhibitor cocktail in accordance with the manufacturer's standard protocol (Sigma Aldrich, UK). Briefly, the growth medium was removed from the cells, then the cells were rinsed once with phosphate buffered saline (PBS), and PBS discarded. 150µl CelLytic M reagent containing 1:100 protease inhibitor cocktail (Sigma Aldrich, UK) were added. Cells were incubated for 15 minutes on a shaker and the lysed cells collected. The lysed cells were centrifuged for 15 minutes at 12000 x g to pellet the cellular debris. The protein-containing supernatant was removed to a chilled tube. Unused lysate was stored at –80°C

Protein was quantified against a BSA standard curve and normalised to 50µg per reaction for analysis of the efficiency of the protein knockdown by Western blot analysis. Western blot was performed in accordance with the manufacturer's instructions (Invitrogen Limited, Paisley, UK). Briefly, 50µg total protein was

combined with an equal volume of NuPage LDS loading buffer and 10% Nupage reducing agent, samples were heated to 70°C for 10 minutes, cooled and loaded onto NuPage Novex Bis-Tris Mini Gels, and run in a XCell Surelock™ Mini Cell at 200V for 50 minutes, the gel was then washed with distilled water and transferred to iBlot nitrocellulose for 7 minutes at 23 volts. The membrane was blocked with 10% non-fat dried milk in 0.1% T-TBS (Tween 20 and Tris buffered saline) overnight at 4°C. The membrane was washed 3 times and the primary antibody added in 2% non-fat dried milk in 0.1% T-TBS for 2 hours at 4°C, washed a further 4 times and the secondary antibody added in 2% non-fat dried milk in 0.1% T-TBS for 1 hour at 4°C. The membrane was washed a further 12 times, blotted and enhanced chemiluminescence performed, exposed to light sensitive film, developed and fixed. Protein bands were validated against Novex Sharp Standards (Invitrogen Limited, Paisley, UK).

3.3.2.8 LXRα Knockdown in the Huh7 Cell Line

Huh7 cells (at passage 22) were transfected with 5µl Lipofectamine 2000/15nM siRNA final concentration (LXRα) and 20nM siRNA GAPDH (positive control) and Non-Targeting (negative control) final concentrations in 6 well Nunclon® Up-Cell® plates for 24h in accordance with the optimised methodology outlined in 3.2.2.7 above. Briefly, the day prior to transfection $2.5x10^6$ cells were plated in 2.5ml 10% FCS/DMEM culture medium so that at transfection they would be 30-50% confluent. The culture media was replaced by pre-warmed (37°C) serum free DMEM media on the day of transfection. LXRα, GAPDH (positive control) and non-targeting (negative control) siRNAs were diluted in 1x siRNA buffer (25µl) (prepared in accordance with Dharmacon instructions) to provide concentrations to give final

reaction concentrations at 15nM (LXRα) and 20nM (positive and negative controls), and incubated for 5 minutes at room temperature whilst the diluted Lipofectamine 2000 in serum free DMEM (25µl) media was prepared. Following incubation the dilute siRNA and dilute Lipofectamine 2000 were combined and incubated at room temperature for a further 20 minutes. The siRNA/Lipofectamine 2000 complexes were added to the appropriate wells containing cells and were mixed gently by rocking the plate. Each well volume was made up to 2.5ml by the addition of a further 2ml serum free DMEM media. The plates were incubated at 37°C and 5% CO_2 for 4 hours. Following 4 hour incubation the siRNA/Lipofectamine 2000 complex containing media was completely aspirated and replaced by pre-warmed (37°C) 10% FCS/DMEM media prior to further incubation at 37°C and 5% CO_2 for 24h until ready for analysis of gene knockdown efficiency by RT-PCR. Efficiency of the protein knockdown was determined by western blot analysis, as described in 3.3.2.7.

3.3.2.9 RT-PCR Gene Expression

Taqman Gene Expression inventoried assays for RT-PCR (Applied Biosystems Limited, Warrington, UK, (Table 2.3)) were used for FXR, LXRα, ApoA-I, ApoC-II, ApoC-III, SREBP-1C, VLDLR, PPARα, GAPDH and β-actin quantified against the housekeeping gene β-2-microglobulin (β2M). Amplification was performed as previously described in Section 2.3.2.9 above.

3.4. Statistical Analysis

Statistical analysis of all results was undertaken using StatsDirect Statistical software version 2.7.8 as previously described in Section 2.4 above.

3.5 Results

3.5.1 MTT Toxicity Assays

Results for the MTT toxicity assays are shown in 2.5.1 of this thesis.

3.5.2 Selection of Preferred Housekeeping Control by Gene Expression Assay

Selection of suitable candidate housekeeping controls was determined by gene expression analysis in Huh7 cells (n=3) in three conditions: untreated cells, mock transfections (Lipofectamine 2000 vehicle with siRNA vehicle only) and siRNA knockdown of LXRα using Taqman Endogenous Control Plates. The results of the initial analysis revealed five potential candidates: GAPDH, which had been identified as a positive control for the siRNA knockdown experiments so was not the preferred housekeeping control; β-actin; HMBS; and RPLPO, Figure 3.1 below; and β-2-microglobulin (β-2M, Figure 3.2). HMBS and RPLPO were not available as large volume endogenous controls in individual Taqman inventoried assays so further analysis was performed using β-actin and β-2M across all treatments in Huh7 cells. β-actin showed greater variance in C_T values across the RTV treated Huh7 cells (Figure 3.3) consequently β-2M (Figure 3.4) was selected as the preferred housekeeping control.

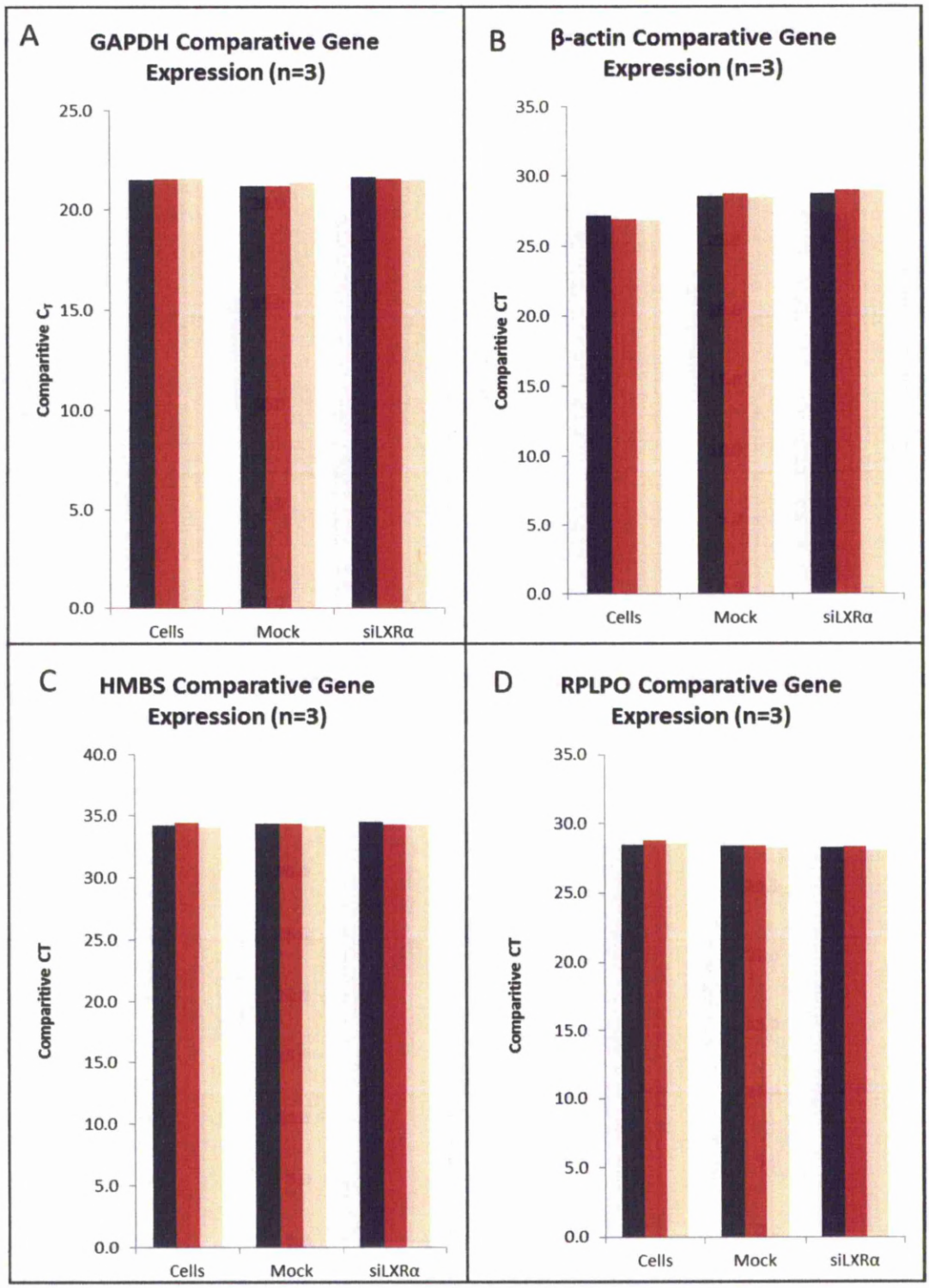


Figure 3.1: Endogenous Control Gene Expression Assay in untreated Huh7, Mock Transfection and siRNA LXRα Knockdown Huh7 cells (p23) n=3 for all assays: A = GAPDH; B = β-actin; C = HMBS; D = RPLPO.

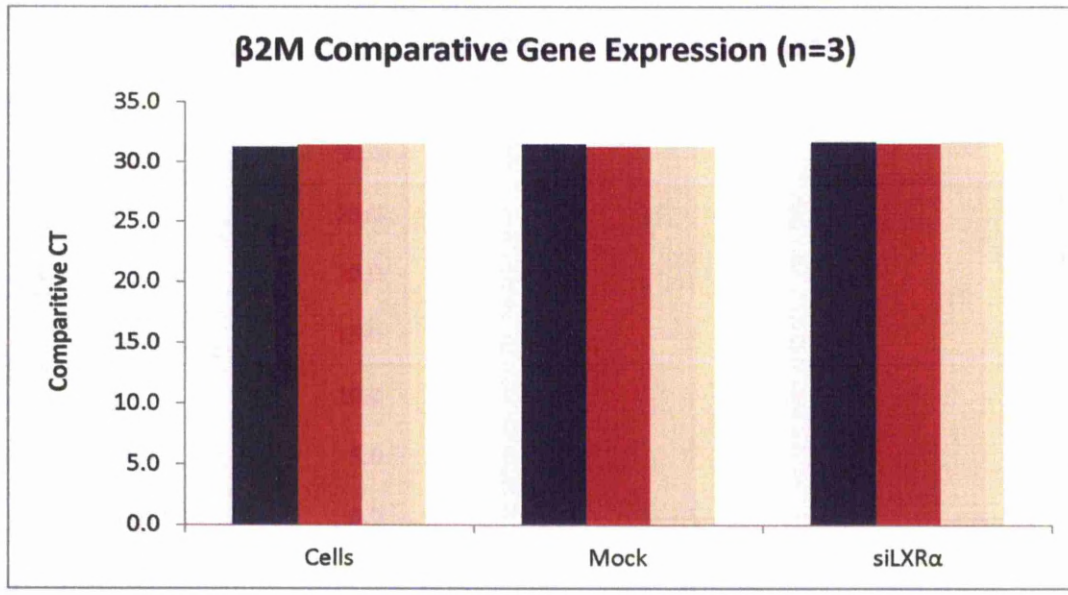


Figure 3.2: β-2-microglobulin Endogenous Control Gene Expression Assay in untreated Huh7, Mock Transfection and siRNA LXRα Knockdown Huh7 cells (p23) n=3.

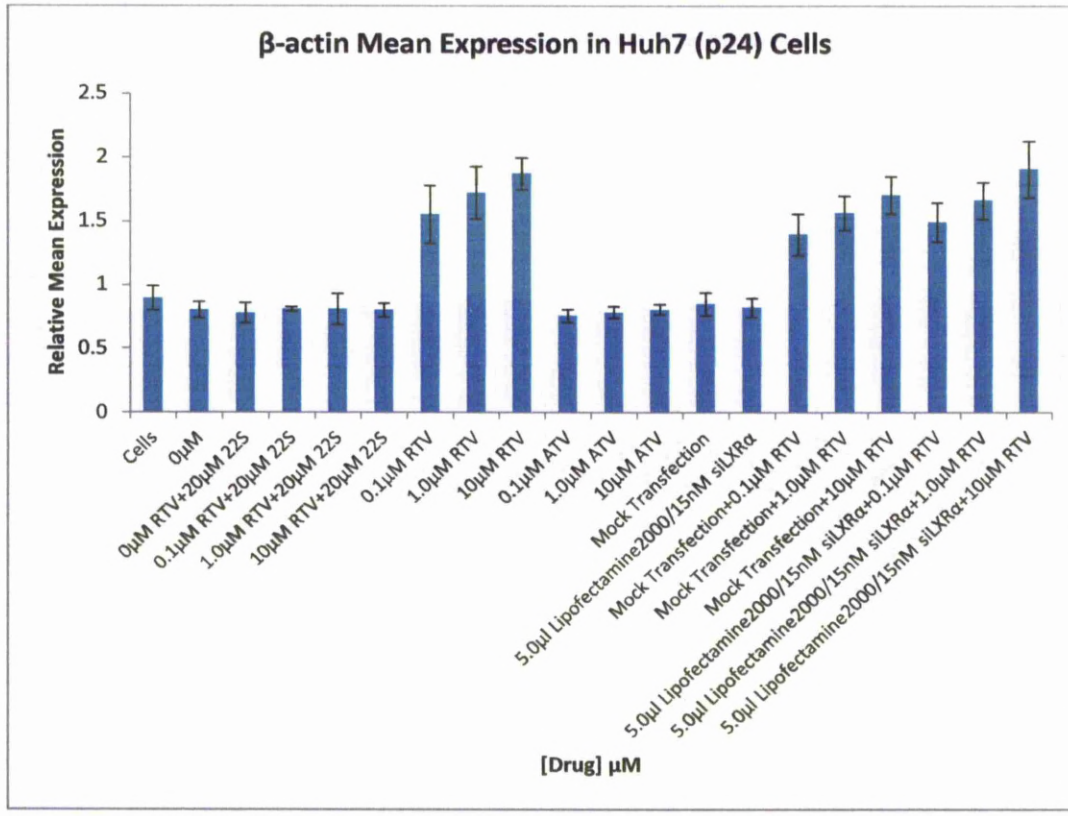


Figure 3.3: β-actin Endogenous Control Gene Expression Assay relative to GAPDH in Huh7 (p24) Cells all conditions (n=4). (Mock Transfection = 5.0μl Lipofectamine 2000/25μl 1 x siRNA buffer (vehicle only) per reaction).

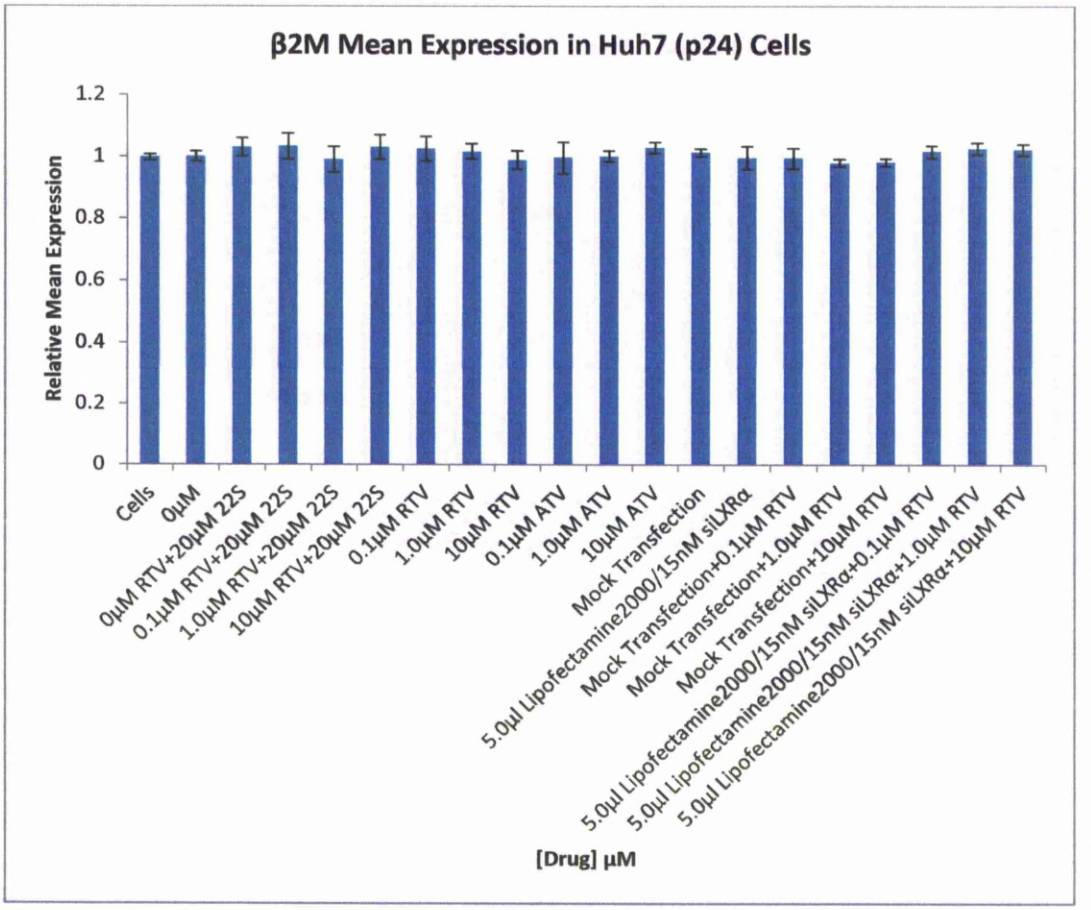


Figure 3.4: β2M Endogenous Control Gene Expression Assay relative to GAPDH in Huh7 (p24) Cells all conditions (n=4). (Mock Transfection = 5.0μl Lipofectamine 2000/25μl 1 x siRNA buffer (vehicle only) per reaction).

3.5.3 Quantification of RNA and cDNA

The RNA extracted from Huh7 cells all conditions as described in Section 3.3.2.4 above, was analysed and quantified using a Thermo-Scientific® Nanodrop 1000 to assess its quantity and purity. The RNA had an A_{260}/A_{280} ratio in the range of 1.85-2.01 (RTV samples), 1.94-2.15 (ATV samples) and 1.85-1.97 (siRNA samples) indicating sufficient purity to proceed to cDNA construction for RT PCR. RNA samples were frozen at -80°C following cDNA construction.

The cDNA for all conditions had A_{260}/A_{280} ratios in the range of 1.98-2.13 indicating sufficient purity to proceed to construction of normalised (20ng/µl) cDNA working stocks. Master and working stock cDNA samples were frozen at -20°C until used in gene expression assays.

3.5.4 Optimisation of LXRα Knockdown in Huh7 Cell Line

Optimal conditions for siRNA knockdown of LXRα, siRNA knockdown of GAPDH (positive control) and non-targeting siRNA (negative control) in Huh7 (passage = 22) cells were determined by evaluating knockdown efficiency of each gene over three time periods: 24h, 48h and 72h, as described in 3.3.2.7 above (Figure 3.5, below). RNA was isolated by the Tri®Reagent method as described in 3.3.2.4 above and reverse transcribed to cDNA as described previously in 3.3.2.5.

Optimum conditions for knockdown were determined to be 1.0µl Lipofectamine 2000 with 15nM LXRα siRNA in 48 well plates at the mRNA level over 24h (Figure 3.5 below) and 48h (Figure 3.5 below), with evidence of recovery at the mRNA level observed after 72h (Figure 3.5 below). Optimal conditions for siRNA knockdown of GAPDH siRNA (Positive control; Figure 3.5 below) and stability of the non-targeting siRNA (negative control; Figure 3.5 below) were determined to be 1.5µl Lipofectamine 2000 with 20nM GAPDH siRNA and 1.5µl Lipofectamine 2000 with 20nM non-targeting siRNA in 48 well plates at the mRNA level over 24h and 48h, mRNA recovery was again observed after 72h.

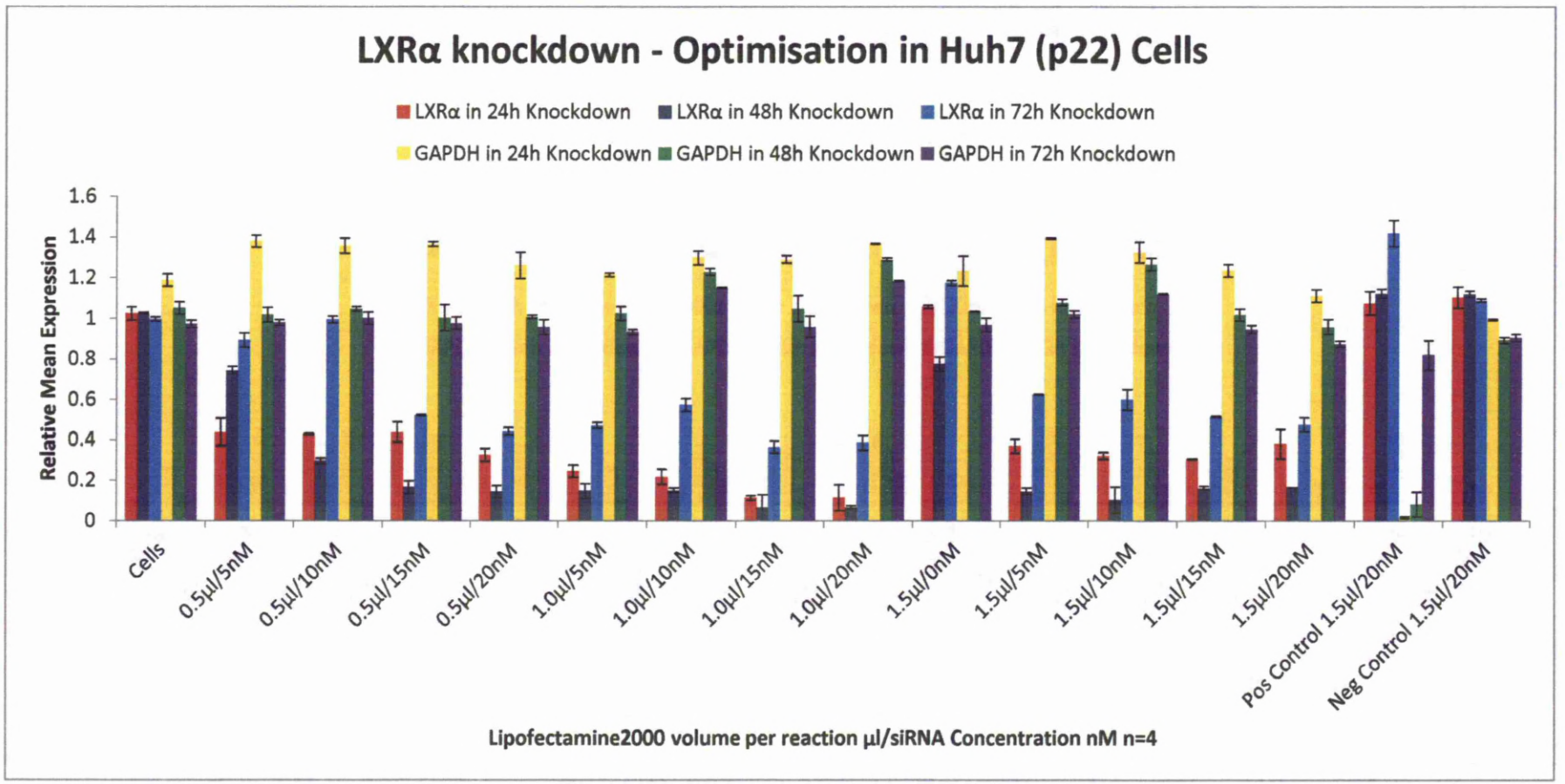


Figure 3.5: Optimisation of LXRα Knockdown in Huh7 cells (passage no. = 22) at 24h, 48h and 72 siRNA knockdown of LXRα and GAPDH (positive control) with Lipofectamine 2000 (0.5-1.5µl) and siRNA (0-20nM) in 48 well plates, by gene expression assay, n=4.

Protein was extracted in accordance with the CelLytic standard protocol and quantified against a BSA standard curve (Figure 3.6, below) as described in 3.3.2.7 above.

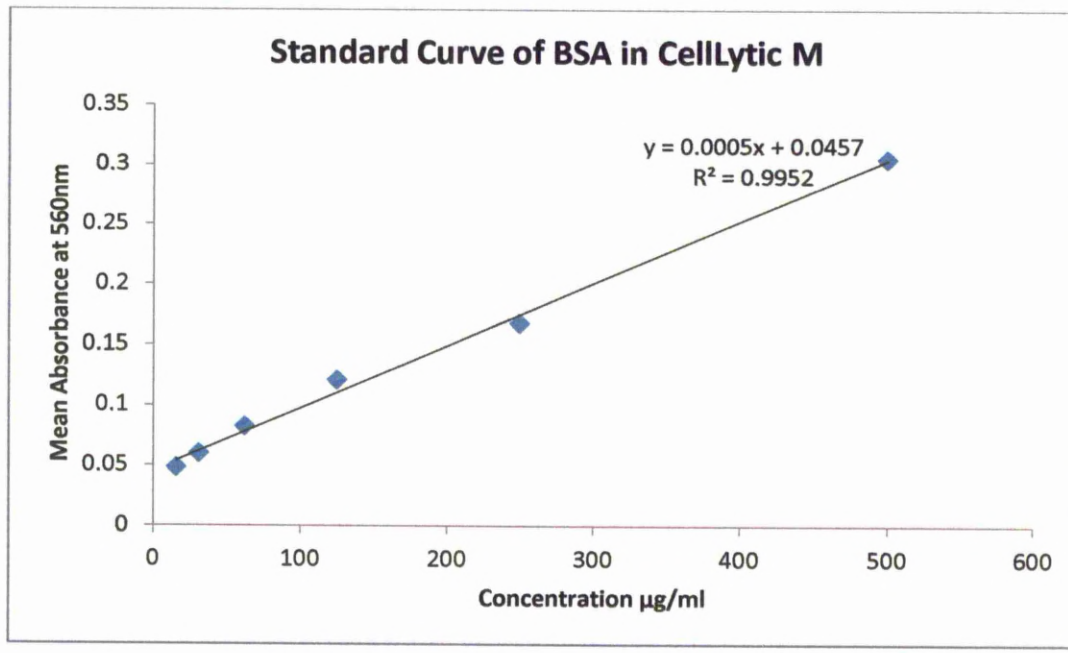


Figure 3.6: Standard Curve of BSA absorbance at 560nm for protein quantification.

Optimal conditions for scaled up siRNA knockdown of LXRα in Huh7 (passage = 23) cells were determined to be 5.0µl Lipofectamine 2000 with 15nM final concentration LXRα siRNA in 6 well Nunclon® Up-Cell® plates at the mRNA level over 24h (Figure 3.7 below). Optimal conditions for siRNA knockdown of GAPDH (Positive control; Figure 3.7 below) and non-targeting siRNAs (negative control; Figure 3.7 below) were determined to be 5.0µl Lipofectamine 2000 with 20nM final concentration GAPDH siRNA and 5.0µl Lipofectamine 2000 with 20nM final concentration non-targeting siRNA in 6 well Nunclon® Up-Cell® at the mRNA level over 24h.

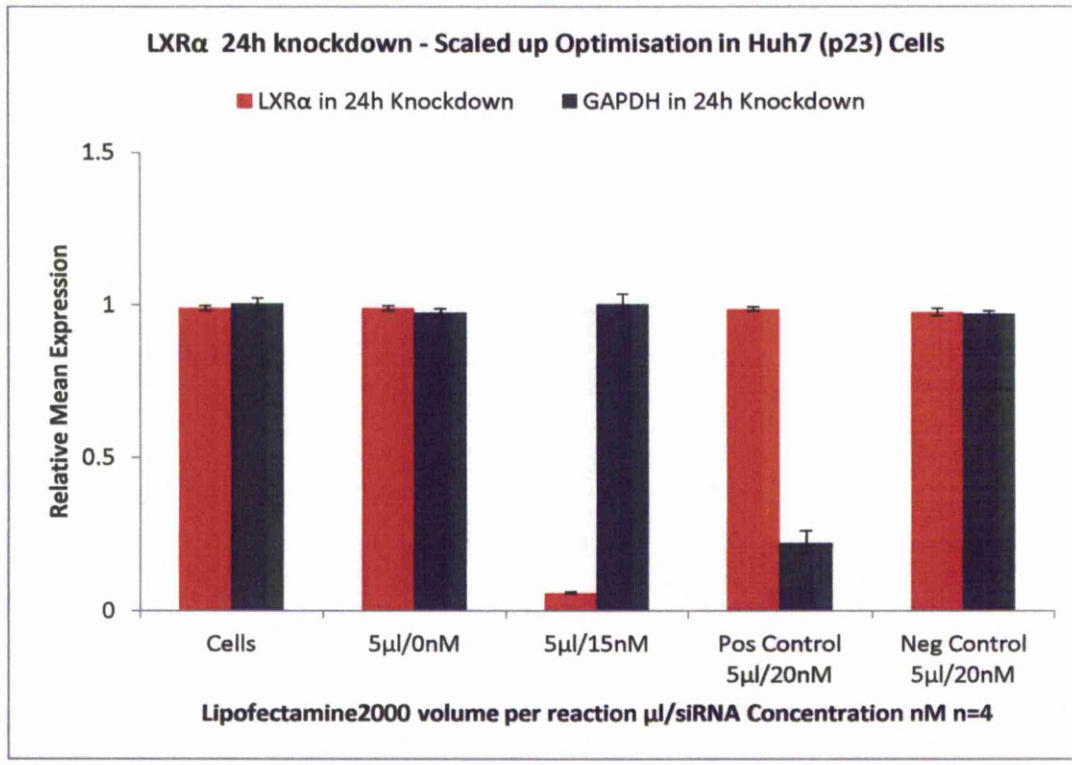


Figure 3.7: Gene Expression Assay in Huh7 cells (passage no. = 23) for optimisation of 24h scaled up siRNA knockdown of LXRα and GAPDH (positive control) in 6 well plates, n=4.

The siRNA knockdown of LXRα and GAPDH at the protein level was confirmed at 24h by Western Blot analysis, see Figure 3.8 below.

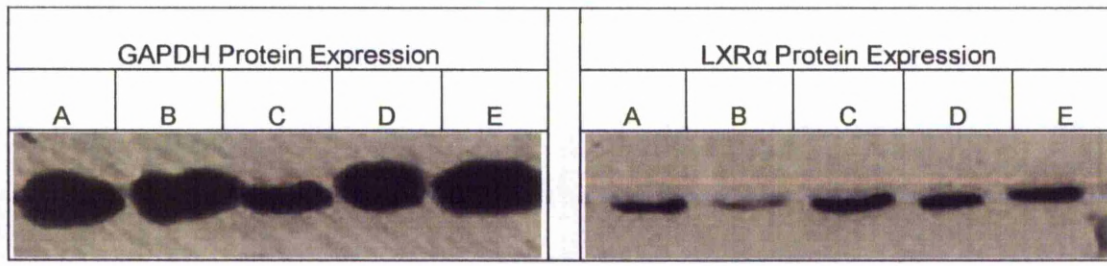


Figure 3.8: Representative GAPDH (Positive Control) and LXRα Protein Expression in 24h siRNA knockdown in Huh7 cells (at passage 23). A = Mock transfection (5µl Lipofectamine 2000/0nM siRNA); B = LXRα Knockdown (5µl Lipofectamine 2000/15nM LXRα siRNA); C = GAPDH Knockdown (5µl Lipofectamine 2000/20nM GAPDH siRNA); D = Negative Control (5µl Lipofectamine 2000/20nM Non-Targeting siRNA); E = Untreated Huh7 Cells.

3.5.5 Gene Expression in Ritonavir Treated LXRα Knockdown Huh7 Cells

Optimal conditions for siRNA knockdown of LXRα in Huh7 cells were determined to be 5.0μl Lipofectamine 2000 with 15nM final concentration LXRα siRNA in 6 well plates at the mRNA and protein levels over 24h (3.3.6 above). Optimal conditions for siRNA knockdown of GAPDH (Positive control) and stable expression of non-targeting siRNA (negative control) were determined to be 5.0μl Lipofectamine 2000 with 20nM GAPDH siRNA and 5.0μl Lipofectamine 2000 with 20nM non-targeting siRNA in 6 well plates at the mRNA and protein levels over 24h (3.5.4 above).

HSP70 gene expression was stable across all concentrations of RTV and ATV in Huh7 cells (n=4), as previously shown in 2.5.6, it was consequently selected as the negative control for the RTV incubation experiments in the siRNA knockdown cells (Figure 3.9 below). There were no statistically significant differences in the HSP70 mean expression values across all conditions compared to vehicle controls, Figure 3.9 below.

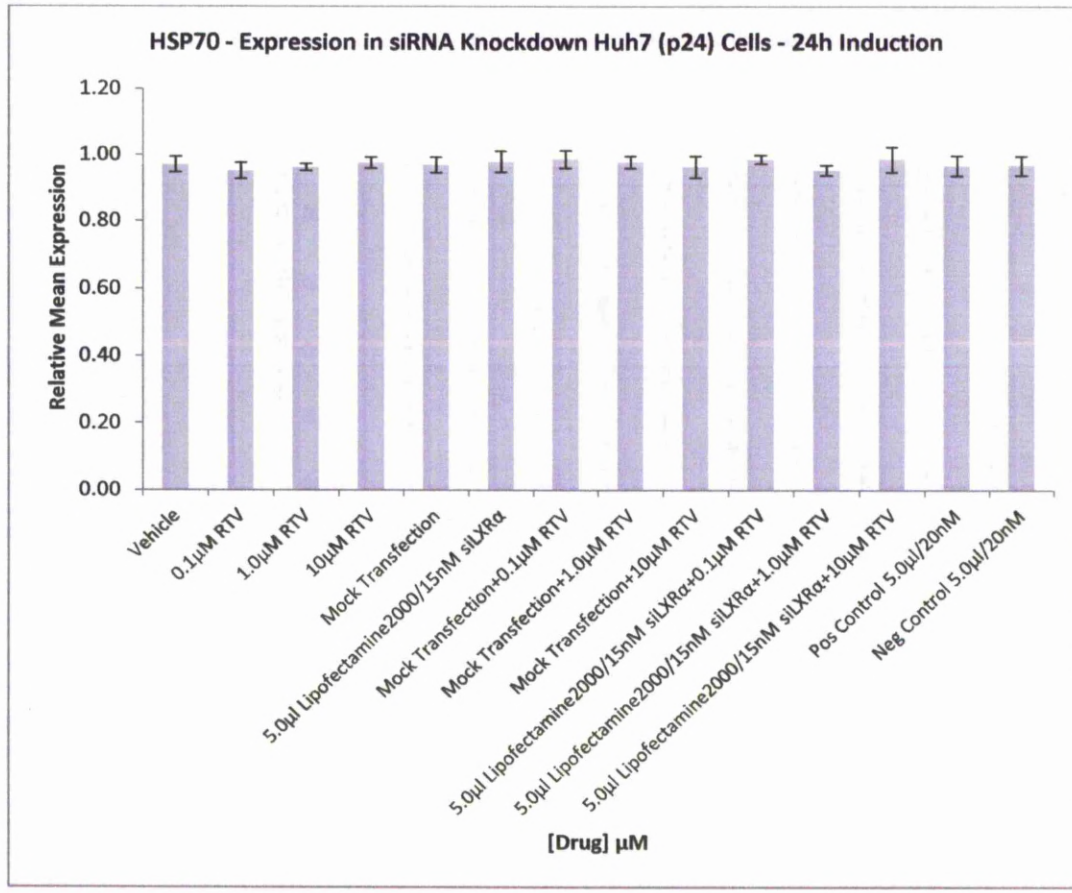


Figure 3.9: HSP70 Gene Expression Assay in Huh7 cells (passage no. = 24) (relative to housekeeping control β-2M) incubated for 24h with ritonavir (RTV), RTV in Mock Transfection Huh7 cells and RTV in 24h siRNA knockdown Huh7 cells, n=4.

Significant up-regulation of ApoA-I by RTV was observed at concentrations of 0.1µM (2.7 fold increase compared to vehicle control, P=<0.0001), 1.0µM (3.6 fold increase compared to vehicle control, P=<0.0001) and 10µM (5.6 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 3.10 below. The statistically significant up-regulation of ApoA-I by RTV was also observed in the Mock Transfection plus RTV incubations at concentrations of 0.1µM RTV (2.7 fold increase compared to vehicle control, P=<0.0001), 1.0µM RTV (3.8 fold

increase compared to vehicle control, P=<0.0001) and 10μM RTV (5.6 fold increase compared to vehicle control, P=<0.0001), Figure 3.10 below. The expression of ApoA-I in siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 3.10 below. The statistically significant up-regulation of ApoA-I by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was abolished in the siRNA LXRα knockdown samples at concentrations of 0.1μM, 1.0μM and 10μM RTV, Figure 3.10 below.

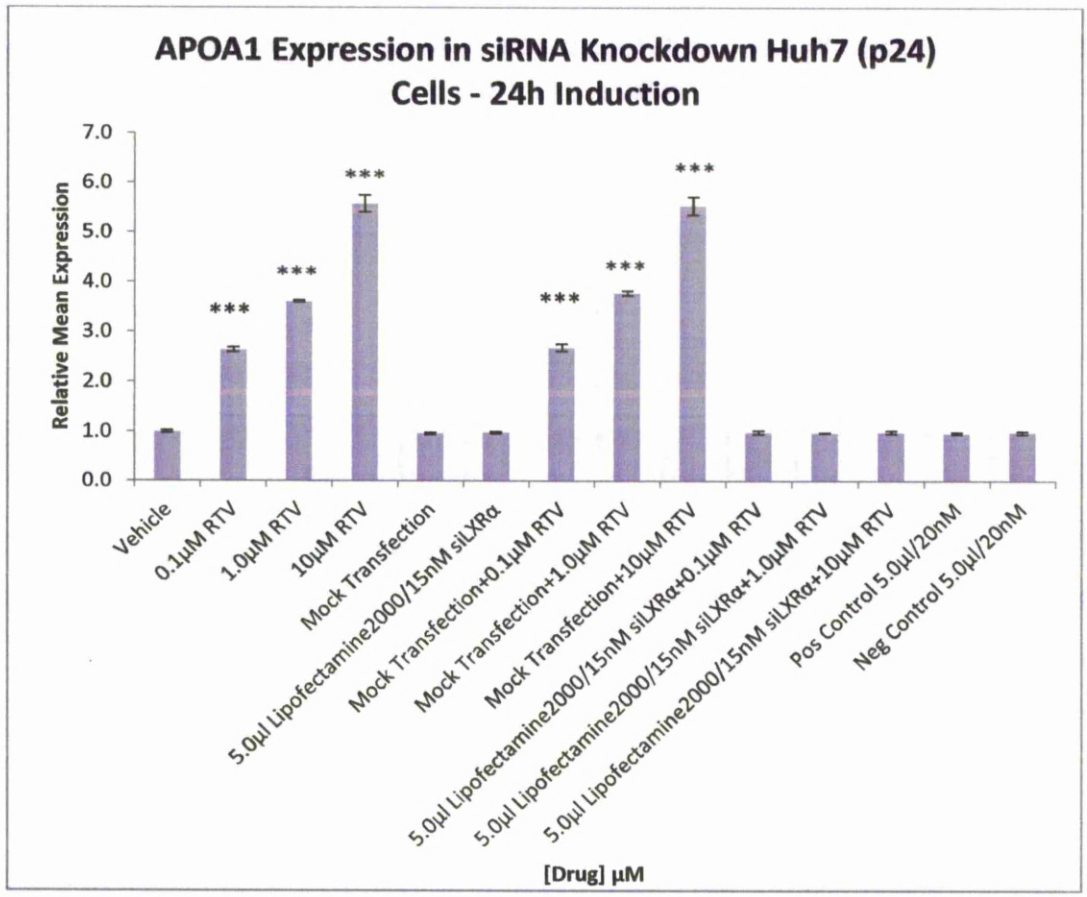


Figure 3.10: APOA Gene Expression Assay in Huh7 cells (passage no. = 24) incubated for 24h with ritonavir (RTV), RTV in Mock Transfection Huh7 cells and RTV in 24h siRNA knockdown Huh7 cells, n=4. *** denotes P=<0.0001.

ApoC-II was significantly up-regulated by RTV at 0.1μM (1.7 fold increase compared to vehicle control, P=<0.0001), 1.0μM (3 fold increase compared to vehicle control, P=<0.0001) and 10μM (9 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 3.11 below. The statistically significant up-regulation of ApoC-II by RTV was also observed in the Mock Transfection plus RTV incubations at concentrations of 0.1μM RTV (1.8 fold increase compared to vehicle control, P=<0.0001), 1.0μM RTV (3.3 fold increase compared to vehicle control, P=<0.0001) and 10μM RTV (8.2 fold increase compared to vehicle control, P=<0.0001), Figure 3.11 below. ApoC-II expression in siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 3.11 below. The statistically significant up-regulation of ApoC-II by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was abolished in the siRNA LXRα knockdown samples at concentrations of 0.1μM, 1.0μM and 10μM RTV, Figure 3.11 below.

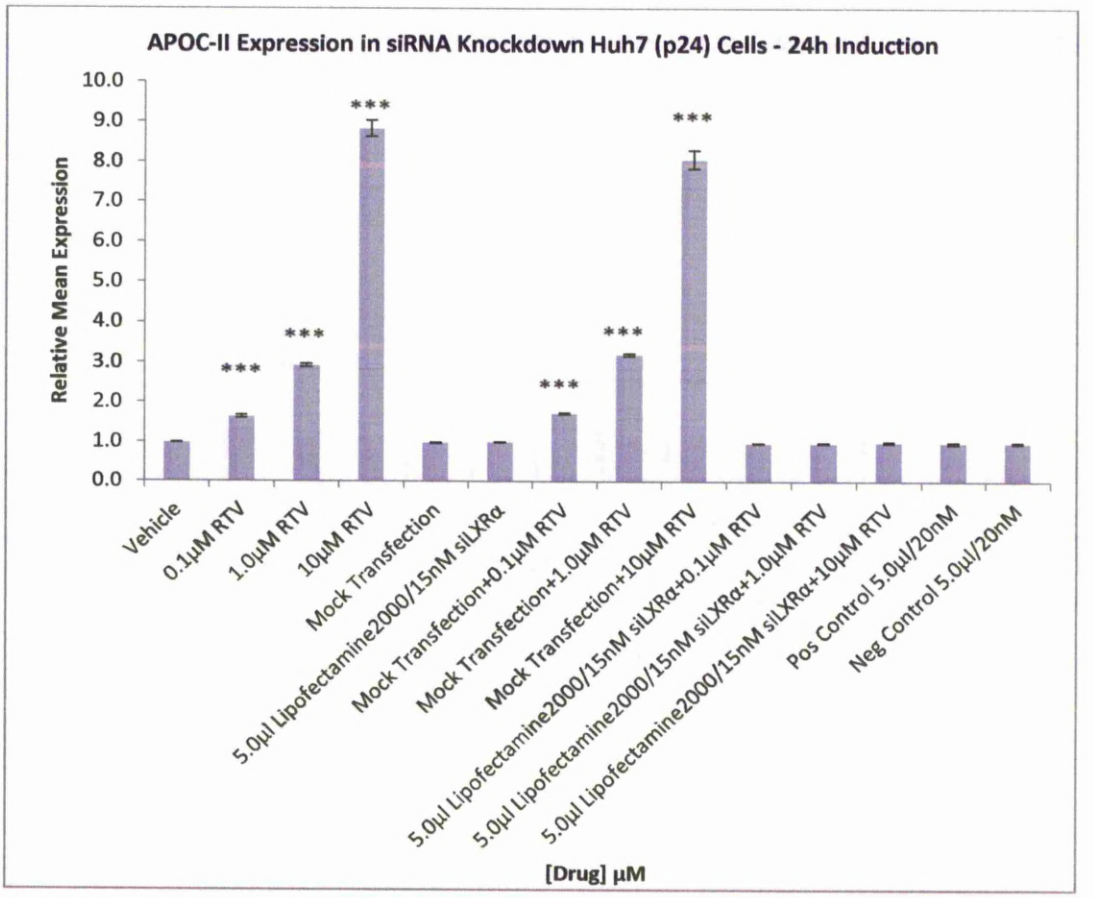


Figure 3.11: ApoC-II Gene Expression Assay in Huh7 cells (passage no. = 24) incubated for 24h with ritonavir (RTV), RTV in Mock Transfection Huh7 cells and RTV in 24h siRNA knockdown Huh7 cells, n=4. *** denotes P=<0.0001.

Significant up-regulation of ApoC-III at 0.1µM (1.2 fold increase compared to vehicle control, P=<0.0001), 1.0µM (1.3 fold increase compared to vehicle control, P=<0.0001) and 10µM RTV was observed (5 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 3.12 below. The significant up-regulation of ApoC-III by RTV was also observed in the Mock Transfection plus RTV incubations at concentrations of 0.1µM RTV (1.2 fold increase compared to vehicle control, P=<0.0001), 1.0µM RTV (1.3 fold increase compared to vehicle control, P=<0.0001) and 10µM RTV (5 fold increase compared to vehicle control,

P=<0.0001), Figure 3.12 below. ApoC-III gene expression in the siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 3.12 below. The statistically significant up-regulation of ApoC-III by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was abolished in the siRNA LXRα knockdown samples at concentrations of 0.1μM, 1.0μM and 10μM RTV, Figure 3.12 below.

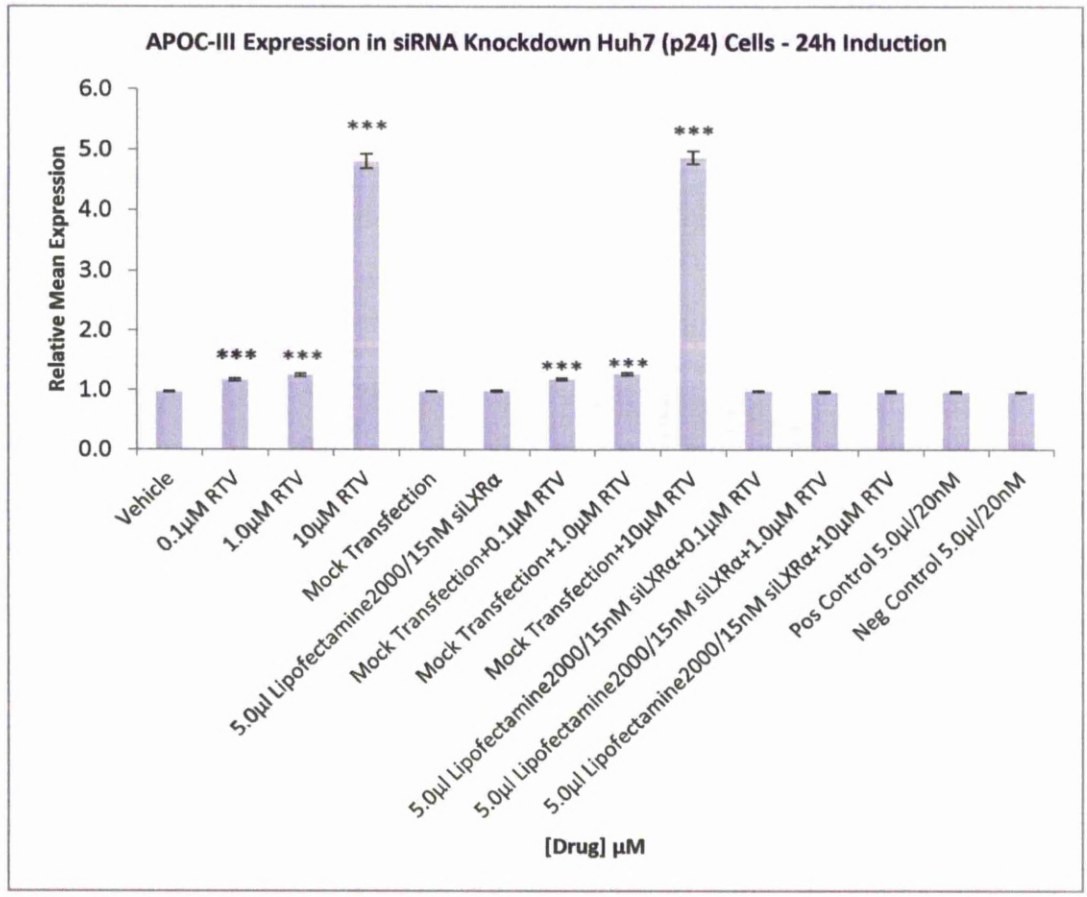


Figure 3.12: ApoC-III Gene Expression Assay in Huh7 cells (passage no. = 24) incubated for 24h with ritonavir (RTV), RTV in Mock Transfection Huh7 cells and RTV in 24h siRNA knockdown Huh7 cells, n=4. *** denotes P=<0.0001.

PPARα gene expression was significantly down-regulated by RTV at concentrations of 0.1μM (2.6 fold decrease compared to vehicle control, P=<0.0001), 1.0μM (5.1

fold decrease compared to vehicle control, P=<0.0001) and 10μM (6.2 fold decrease compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 3.13 below. A statistically significant down-regulation of PPARα gene expression by RTV was also observed in the Mock Transfection plus RTV incubations at concentrations of 0.1μM RTV (2.4 fold decrease compared to vehicle control, P=<0.0001), 1.0μM RTV (4.9 fold decrease compared to vehicle control, P=<0.0001) and 10μM RTV (6.3 fold decrease compared to vehicle control, P=<0.0001), Figure 3.13 below. The expression of PPARα in the siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 3.13 below. The statistically significant down-regulation of PPARα gene expression by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was abolished in the siRNA LXRα knockdown samples at concentrations of 0.1μM, 1.0μM and 10μM RTV, Figure 3.13 below.

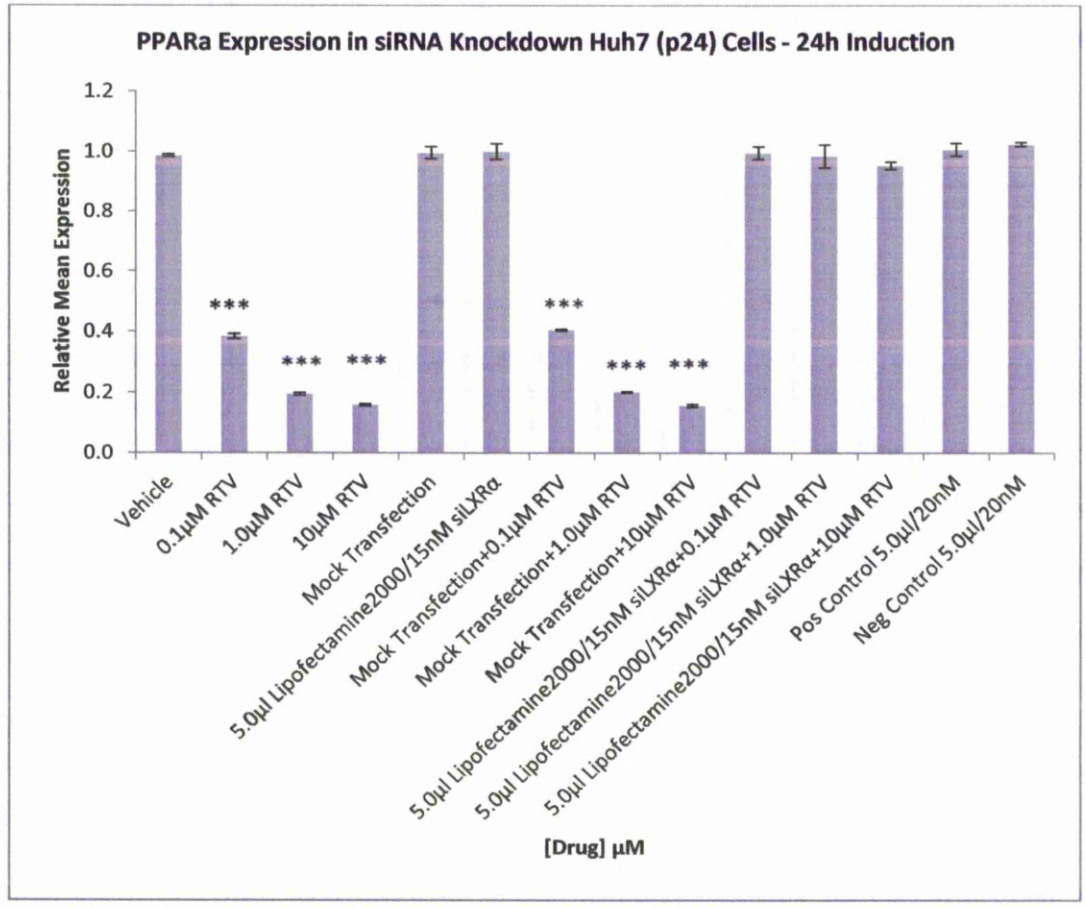


Figure 3.13: PPARα Gene Expression Assay in Huh7 cells (passage no. = 24) incubated for 24h with ritonavir (RTV), RTV in Mock Transfection Huh7 cells and RTV in 24h siRNA knockdown Huh7 cells, n=4. *** denotes P=<0.0001.

Significant up-regulation of SREBP-1c at 0.1μM (1.3 fold increase compared to vehicle control, P=<0.0001), 1.0μM (2.1 fold increase compared to vehicle control, P=<0.0001) and 10μM RTV was observed (3.2 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 3.14 below. The statistically significant up-regulation of SREBP-1c by RTV was also observed in the Mock Transfection plus RTV incubations at concentrations of 0.1μM RTV (1.3 fold increase compared to vehicle control, P=0.0003), 1.0μM RTV (2 fold increase compared to vehicle control, P=<0.0001) and 10μM RTV (3.2 fold increase

compared to vehicle control, P=<0.0001), Figure 3.14 below. The expression of SREBP-1c in the siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 3.14 below. The statistically significant up-regulation of SREBP-1c by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was abolished in the siRNA LXRα knockdown samples at concentrations of 0.1μM, 1.0μM and 10μM RTV, Figure 3.14 below.

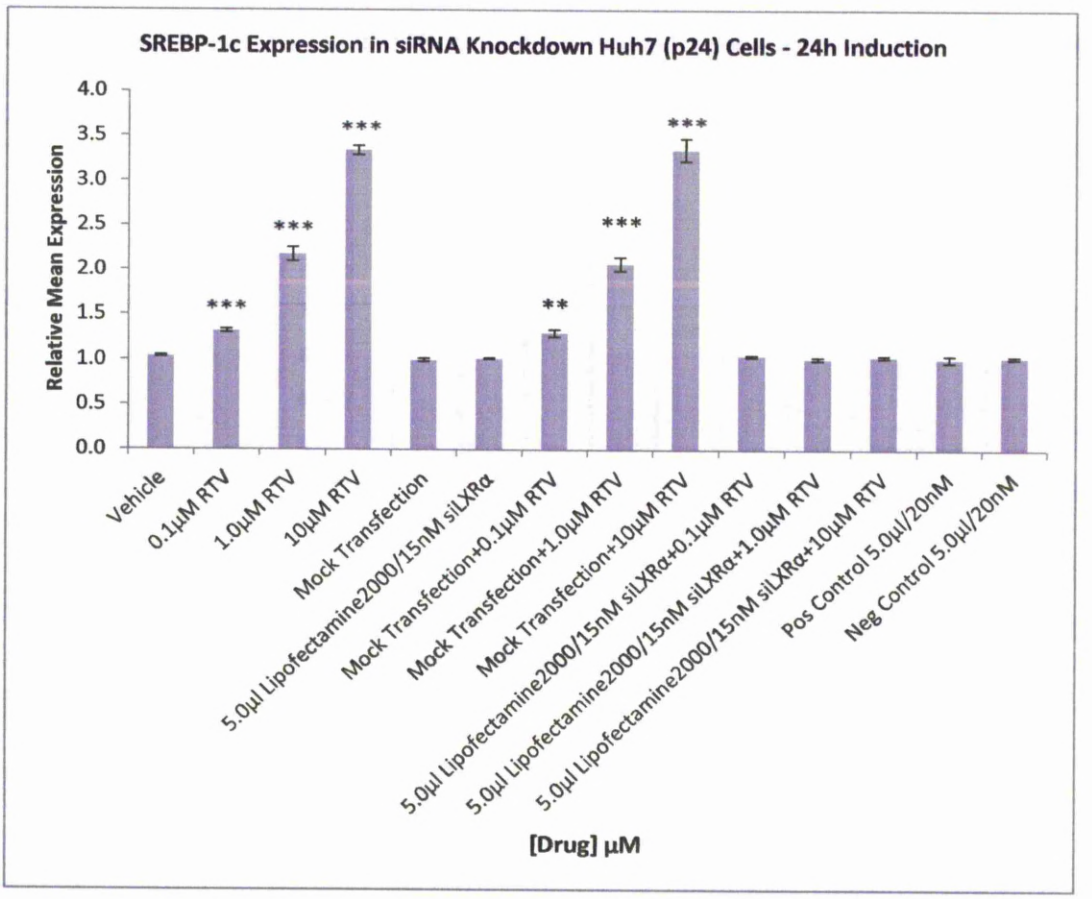


Figure 3.14: SREBP-1c Gene Expression Assay in Huh7 cells (passage no. = 24) incubated for 24h with ritonavir (RTV), RTV in Mock Transfection Huh7 cells and RTV in 24h siRNA knockdown Huh7 cells, n=4. *** denotes P=<0.0001, ** denotes P=<0.001.

VLDLR was significantly down-regulated by RTV at concentrations of 0.1μM (7.7 fold decrease compared to vehicle control, P=<0.0001), 1.0μM (15.8 fold decrease compared to vehicle control, P=<0.0001) and 10μM (10.9 fold decrease compared to vehicle control, P=<0.0001), in Huh7 cells (n=4), Figure 3.15 below. The statistically significant down-regulation of VLDLR gene expression by RTV was also observed in the Mock Transfection plus RTV incubations at concentrations of 0.1μM RTV (8.2 fold decrease compared to vehicle control, P=<0.0001), 1.0μM RTV (14.6 fold decrease compared to vehicle control, P=<0.0001) and 10μM RTV (13 fold decrease compared to vehicle control, P=<0.0001), Figure 3.15 below. The expression of VLDLR in the siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 3.15 below. The statistically significant down-regulation of VLDLR gene expression by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was diminished in the siRNA LXRα knockdown samples at concentrations of 0.1μM, 1.0μM and 10μM RTV but remained statistically significant. At concentrations of 0.1μM RTV in the siRNA LXRα knockdown cells (1.2 fold decrease (reduced from 8.2 fold decrease in mock transfection) compared to vehicle control, P=0.0008), 1.0μM RTV in the siRNA LXRα knockdown cells (1.2 fold decrease (reduced from 14.6 fold decrease in mock transfection) compared to vehicle control, P=0.0002) and 10μM RTV in the siRNA LXRα knockdown cells (1.2 fold decrease (reduced from 13 fold decrease in mock transfection) compared to vehicle control, P=0.0004) in Huh7 cells (n=4), Figure 3.15 below.

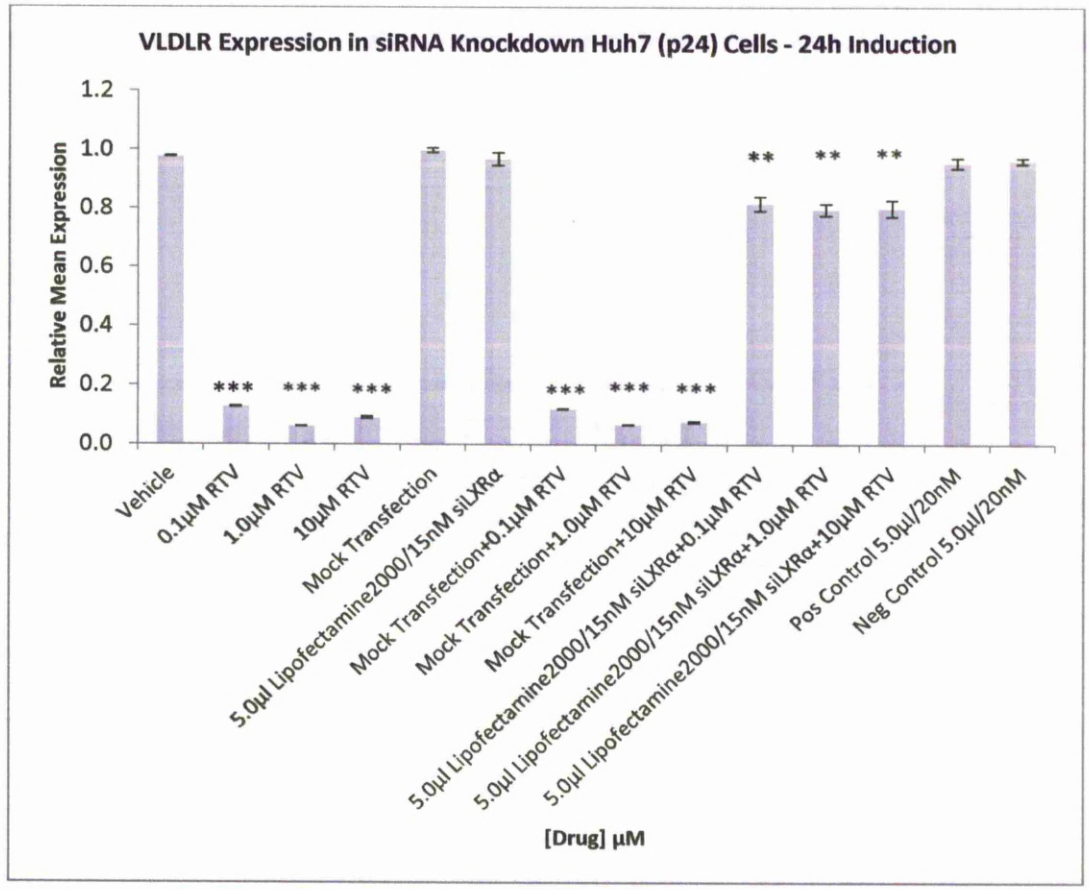


Figure 3.15: VLDLR Gene Expression Assay in Huh7 cells (passage no. = 24) incubated for 24h with ritonavir (RTV), RTV in Mock Transfection Huh7 cells and RTV in 24h siRNA knockdown Huh7 cells, n=4. *** denotes P=<0.0001, ** denotes P=<0.001.

LXRα was also significantly up-regulated by RTV concentrations of 0.1μM (3.8 fold increase compared to vehicle control, P=<0.0001), 1.0μM (6.7 fold increase compared to vehicle control, P=<0.0001) and 10μM (16.7 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 3.16 below. The statistically significant up-regulation of LXRα by RTV was also observed in the Mock Transfection plus RTV incubations at concentrations of 0.1μM RTV (4.5 fold increase compared to vehicle control, P=<0.0001), 1.0μM RTV (6.9 fold increase compared to vehicle control, P=<0.0001) and 10μM RTV (16.9 fold increase

compared to vehicle control, P=<0.0001), Figure 3.16 below. The expression of LXRα in the siRNA LXRα knockdown cells without RTV was significantly decreased from basal expression (8 fold decrease compared to vehicle control, P=<0.0001), Figure 3.16 below. The statistically significant up-regulation of LXRα gene expression by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was completely diminished in the siRNA LXRα knockdown samples at concentrations of 0.1μM, 1.0μM and 10μM RTV. However, LXRα gene expression levels remained repressed compared to vehicle controls at all RTV concentrations in the siRNA LXRα knockdown cells. This repression of LXRα gene expression was statistically significant at concentrations of 0.1μM RTV in the siRNA LXRα knockdown cells (4.5 fold decrease compared to vehicle control, P=<0.0001), 1.0μM RTV in the siRNA LXRα knockdown cells (4.2 fold decrease compared to vehicle control, P=<0.0001) and 10μM RTV in the siRNA LXRα knockdown cells (3.8 fold decrease compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 3.16 below.

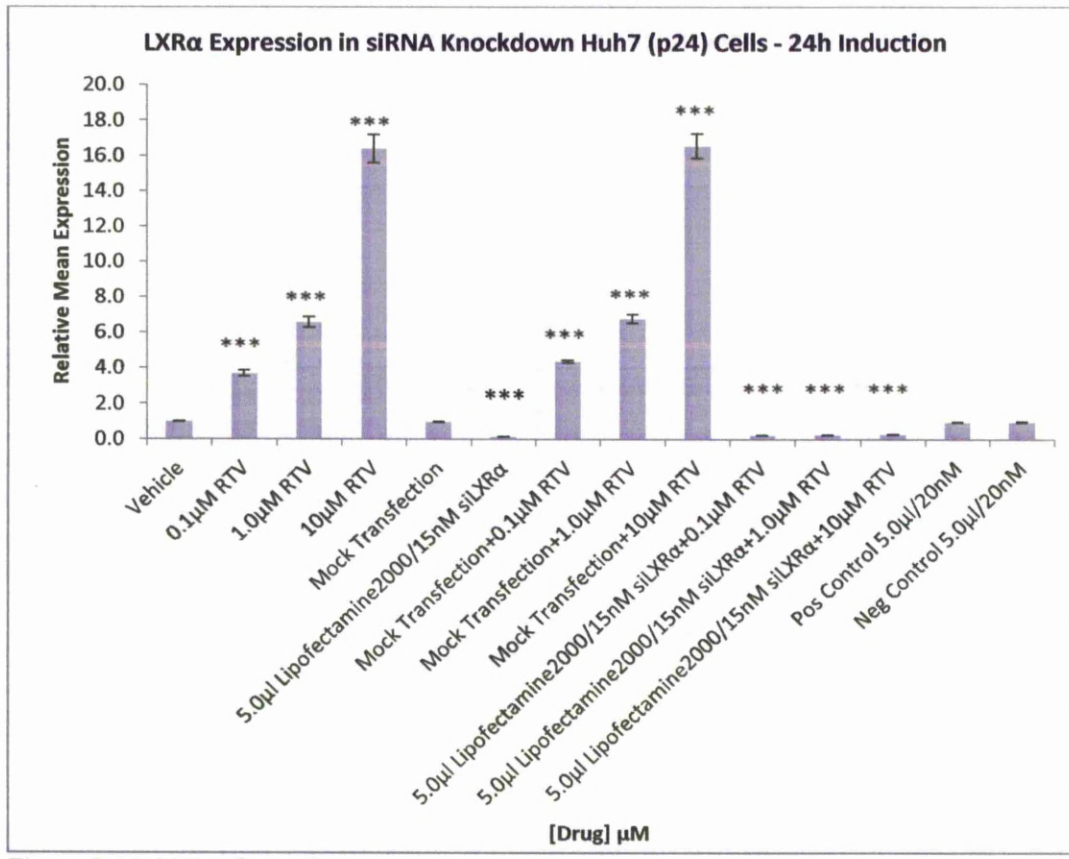


Figure 3.16: LXRα Gene Expression Assay in Huh7 cells (passage no. = 24) incubated for 24h with ritonavir (RTV), RTV in Mock Transfection Huh7 cells and RTV in 24h siRNA knockdown Huh7 cells, n=4. *** denotes P=<0.0001.

HNF4α was also significantly down-regulated by RTV at concentrations of 0.1μM (1.4 fold decrease compared to vehicle control, P=<0.0001), 1.0μM (2.8 fold decrease compared to vehicle control, P=<0.0001) and 10μM (5.3 fold decrease compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 3.17 below. HNF4α, however, was also significantly down-regulated by RTV in the Mock Transfection plus RTV incubations at concentrations of 0.1μM RTV (1.4 fold decrease compared to vehicle control, P=<0.0001), 1.0μM RTV (2.5 fold decrease compared to vehicle control, P=<0.0001) and 10μM RTV (5.4 fold decrease compared to vehicle control, P=<0.0001), Figure 3.17 below. The expression of

HNF4α in the untreated siRNA LXRα knockdown cells was not significantly altered from basal expression levels in the vehicle controls, Figure 3.17 below. The statistically significant down-regulation of HNF4α by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was also observed in the siRNA LXRα knockdown samples at concentrations of 0.1μM RTV (1.3 fold decrease compared to vehicle control, P=<0.0001), 1.0μM RTV (2.7 fold decrease compared to vehicle control, P=<0.0001) and 10μM RTV (5.1 fold decrease compared to vehicle control, P=<0.0001), Figure 3.17 below.

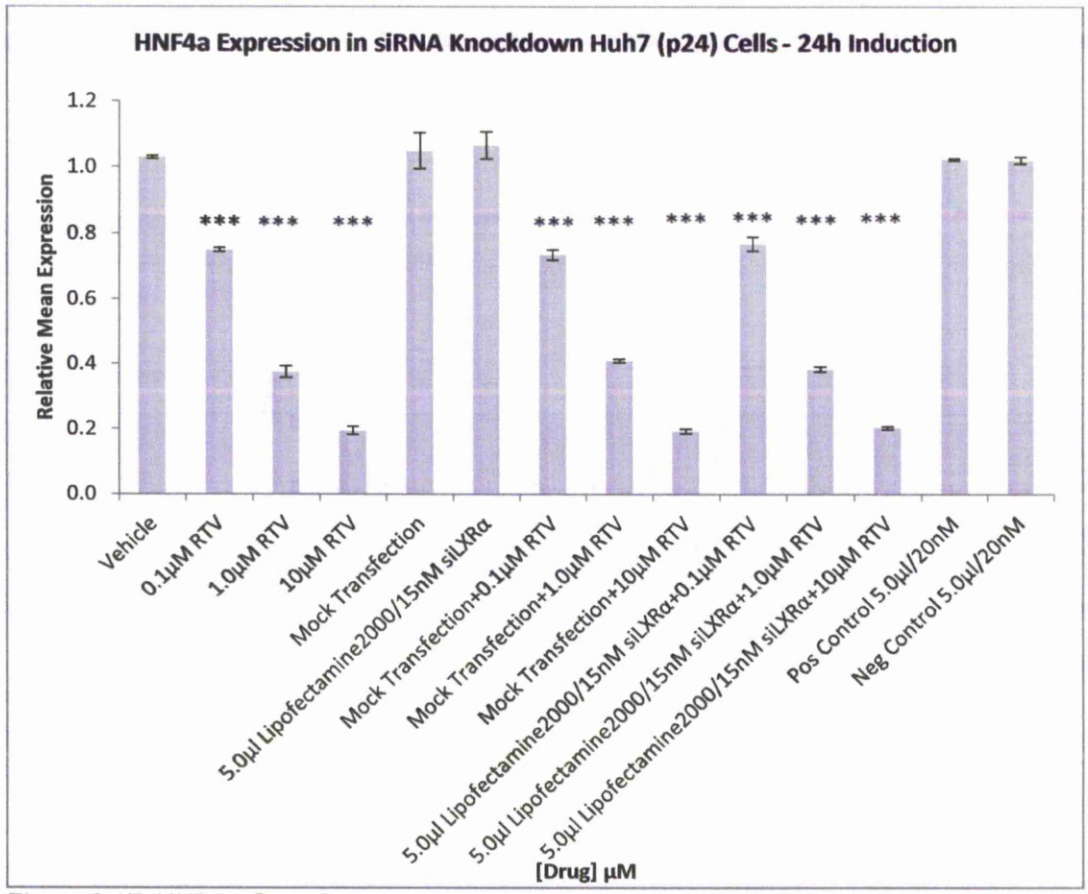


Figure 3.17: HNF4α Gene Expression Assay in Huh7 cells (passage no. = 24) incubated for 24h with ritonavir (RTV), RTV in Mock Transfection Huh7 cells and RTV in 24h siRNA knockdown Huh7 cells, n=4. *** denotes P=<0.0001.

FXR was also significantly up-regulated by RTV concentrations of 0.1μM (1.5 fold increase compared to vehicle control, P=<0.0001), 1.0μM (2.1 fold increase compared to vehicle control, P=<0.0001) and 10μM (1.5 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 3.18 below. The statistically significant up-regulation of FXR by RTV was also observed in the Mock Transfection plus RTV incubations at concentrations of 0.1μM RTV (1.5 fold increase compared to vehicle control, P=<0.0001), 1.0μM RTV (2.1 fold increase compared to vehicle control, P=<0.0001) and 10μM RTV (1.9 fold increase compared to vehicle control, P=<0.0001), Figure 3.18 below. The expression of FXR in the siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 3.18 below. The statistically significant up-regulation of FXR by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was also observed in siRNA LXRα knockdown samples at concentrations of 0.1μM RTV (1.6 fold increase compared to vehicle control, P=<0.0001), 1.0μM RTV (2 fold increase compared to vehicle control, P=<0.0001) and 10μM RTV (1.7 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 3.18 below.

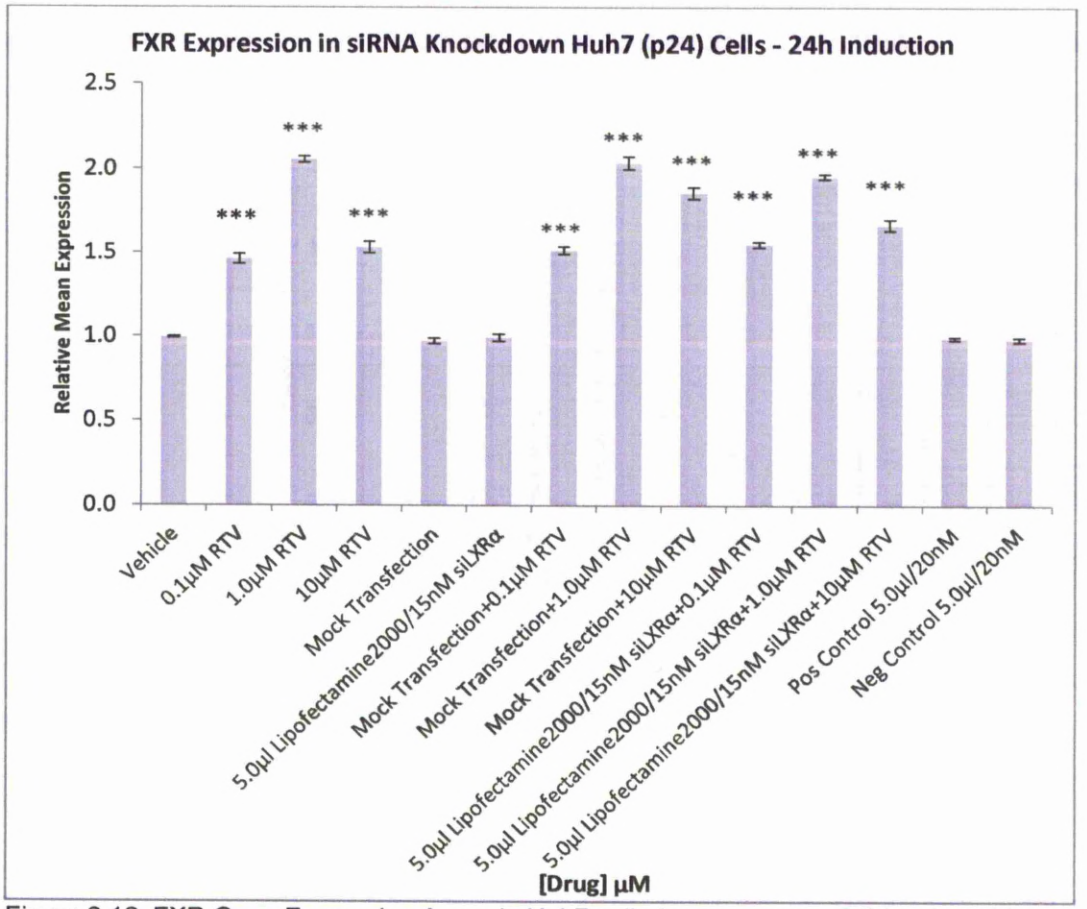


Figure 3.18: FXR Gene Expression Assay in Huh7 cells (passage no. = 24) incubated for 24h with ritonavir (RTV), RTV in Mock Transfection Huh7 cells and RTV in 24h siRNA knockdown Huh7 cells, n=4. *** denotes P=<0.0001.

VDR was also significantly up-regulated by RTV concentrations of 0.1µM (1.5 fold increase compared to vehicle control, P=<0.0001), 1.0µM (2 fold increase compared to vehicle control, P=<0.0001) and 10µM (7.3 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 3.19 below. The statistically significant up-regulation of VDR by RTV was also observed in the Mock Transfection plus RTV incubations at concentrations of 0.1µM RTV (1.6 fold increase compared to vehicle control, P=<0.0001), 1.0µM RTV (2.1 fold increase compared to vehicle control, P=<0.0001) and 10µM RTV (7.9 fold increase

compared to vehicle control, P=<0.0001), Figure 3.19 below. The expression of VDR in siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 3.19 below. The statistically significant up-regulation of VDR by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was also observed in the siRNA LXRα knockdown samples at concentrations of 0.1μM RTV (1.5 fold increase compared to vehicle control, P=<0.0001), 1.0μM RTV (2.1 fold increase compared to vehicle control, P=<0.0001) and 10μM RTV (8.5 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 3.19 below.

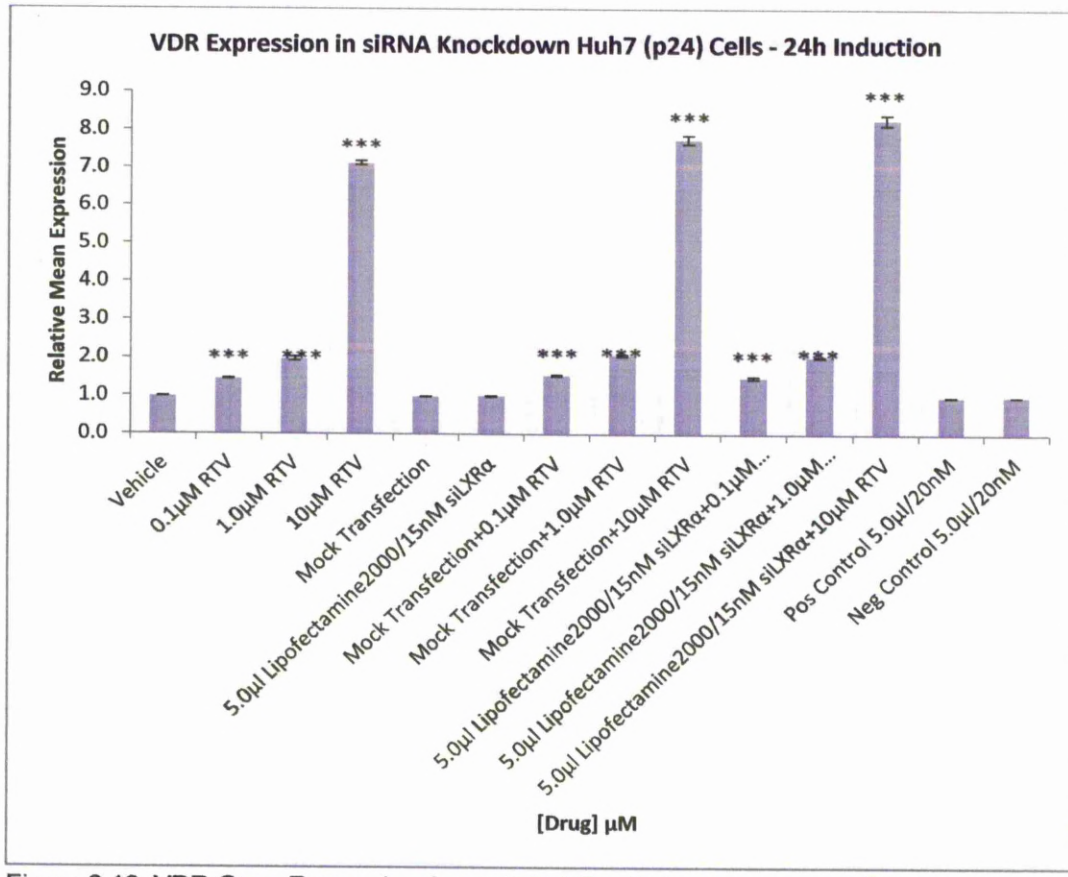


Figure 3.19: VDR Gene Expression Assay in Huh7 cells (passage no. = 24) incubated for 24h with ritonavir (RTV), RTV in Mock Transfection Huh7 cells and RTV in 24h siRNA knockdown Huh7 cells, n=4. *** denotes P=<0.0001.

3.6 Discussion

The inhibition of LXRα gene expression by a chemical antagonist was discussed in Chapter 2. The implications the inhibition of LXRα gene expression had on downstream target genes was also discussed together with the hypothesis that LXRα functions as a master regulator of this downstream gene expression. In this chapter, the findings of Chapter 2 were further probed to validate the role of LXRα and to confirm this hypothesis. The specific focus of this chapter was to determine the impact of the PI RTV on gene expression in NRs involved in lipid metabolism identified in Section 2.1 of this thesis, and on the gene expression of downstream targets in their metabolic pathways (Table 2.2), thereby determining the influence of LXRα. Probing the influence of LXRα knockdown by siRNA on its own gene expression and that of its target genes confirmed the effects of LXRα on gene expression in the presence of RTV and RTV plus 22(S)-hydroxycholesterol seen in Section 2.5.6 and validated the observed role of LXRα on gene expression in response to RTV.

The observed significant up-regulation of ApoC-II ($P=<0.0001$ at 0.1μM, 1μM and 10μM) and significant down-regulation of HNF4α ($P=<0.0001$ at 0.1μM, 1μM and 10μM) gene expression was consistent with the expected outcome of the significant induction of FXR ($P=<0.0001$ at 0.1μM, 1μM and 10μM) by RTV. As previously seen in Section 2.5.6, the addition of the LXRα antagonist 22(S)-hydroxycholesterol did not knock-out the up-regulation of FXR or the down-regulation of HNF4α by RTV. Biological knockdown of LXRα by siRNA in the RTV incubation experiments

did not knock-out the up-regulation of FXR or the down-regulation of HNF4α by RTV, which confirmed the earlier findings and supported the hypothesis that LXRα is not a master regulator of these genes. The up-regulation of ApoC-II by RTV was, however, completely abolished in the biological knockdown of LXRα samples, confirming the observed results with the LXRα antagonist, Section 2.5.6. This supported the hypothesis that more than one mechanism is involved in the up-regulation of ApoC-II and that LXRα is a key regulator of its gene expression.

The RTV incubation assays resulted in significant up-regulation of SREBP-1c (P=<0.0001 at 0.1μM, 1μM and 10μM). This was inconsistent with the concentration dependent significant increase in FXR expression, which suggested another mechanism was involved with SREBP-1c up-regulation. It has previously been reported that the effects of LXRα on fatty acid synthesis are mediated through increased expression of the transcription factor , SREBP-1c, and that SREBP-1c has been associated with PI induced lipodystrophy (Telenti *et al.*, 2002). As significant up-regulation of LXRα was observed at all RTV concentrations (P=<0.0001 at 0.1μM, 1μM and 10μM, with maximal 16.7 fold up-regulation at 10μM RTV), it is likely that LXRα mediated the up-regulation of SREBP-1c in these assays. The inhibition of LXRα by the antagonist 22(S)-hydroxycholesterol and the abolition of its effects on SREBP-1c were demonstrated in Section 2.5.6. These findings were confirmed by biological knockdown of LXRα by siRNA which also abolished the induction effects of RTV on both LXRα and SREBP-1c and supported this hypothesis. Biological knockdown by siRNA of LXRα actually resulted in down-regulation of LXRα gene expression in the presence of all RTV concentrations

(P=<0.0001 at 0.1μM, 1μM and 10μM, with maximal 4.5 fold down-regulation at 0.1μM RTV). This could provide a potential future therapeutic mechanism for the inhibition of LXRα *in vivo,* as interest develops in the use of siRNA for gene therapy for complex metabolic disorders. This opportunity is discussed further in Chapter 7.

It is hypothesised that LXRα is involved in the down-regulation of PPARα gene expression which was significantly down-regulated at all concentrations (P=<0.0001 at 0.1μM, 1μM and 10μM) in the RTV assays. In Chapter 2, inhibition of LXRα by the chemical antagonist 22(S)-hydroxycholesterol was shown to abolish these effects. The use of siRNA to biologically knockdown LXRα confirmed the hypothesis that LXRα is a key player in the regulation of PPARα as the effects of RTV on PPARα were also abolished in these assays.

In Chapter 2, the expected outcome of significant up-regulation of VDR, like FXR, by RTV was discussed. The up-regulation of these two genes should have resulted in down-regulation of ApoA-I gene expression, but significant up-regulation was observed at all RTV concentrations (P=<0.0001 at 0.1μM, 1μM and 10μM). This supported the hypothesis that up-regulation of LXRα target genes may have been the mechanism involved with the up-regulation of ApoA-I. When RTV was co-incubated with the LXRα antagonist, 22(S)-hydroxycholesterol, the up-regulation of ApoA-I was abolished. This was confirmed in the assays incubated with RTV following biological knockdown of LXRα by siRNA, supporting the hypothesis.

It has been reported that PPARα induction leads to the repression of ApoC-III (Duval *et al.*, 2007; Feldman *et al.*, 2008; Yamada *et al.*, 2007). In the RTV assays, the observed significant down-regulation of PPARα may have contributed to the significant induction of ApoC-III, (a 5 fold increase compared to the vehicle control at 10μM RTV, P=<0.0001), as LXRα induces ApoC-III through its repression of PPARα, which was significantly down-regulated at all RTV concentrations. Chemical inhibition of LXRα by 22(S)-hydroxycholesterol was shown to abolish the effects of RTV on ApoC-III in Section 2.5.6 in support of the hypothesis. This was validated by biological knockdown of LXRα by siRNA which also abolished the effects of RTV on ApoC-III (and PPARα) confirming the hypothesis that LXRα is a key player in the regulation of both ApoC-III and PPARα.

LXRα also inhibits VLDLR gene expression which was significantly down-regulated at all RTV concentrations (P=<0.0001 at 0.1μM, 1μM and 10μM) with the maximal 15.8 fold decrease compared to vehicle seen at 1.0μM RTV. These effects can be abolished by the inhibition of LXRα the chemical antagonist 22(S)-hydroxycholesterol as shown in Section 2.5.6, which supported the hypothesis that LXRα was regulating VLDLR. The biological knockdown of LXRα by siRNA underpinned this hypothesis as the impact of RTV on VLDLR gene expression was diminished to a 1.2 fold decrease at all RTV concentrations compared to vehicle controls, although this decrease in VLDLR expression remained statistically significant, suggesting that other mechanism may be involved in the regulation of VLDLR by RTV.

The Liverpool HIV Pharmacology Group is researching the interplay between intracellular accumulation of ARVs and metabolism, and its relevance to the induction of drug disposition genes in HIV therapy with a specific focus on the role of FXR, LXRα and PPARα in the metabolic complications associated with the use of these drugs. This thesis has principally focussed on the role of LXRα in these complications. Polymorphisms in LXRα and key metabolic target genes were also investigated to establish the clinical relevance of these findings (Chapters 5 and 6).

In summary, this chapter has described the comparative effects of biological knockout of LXRα on the differing gene expression profiles associated with RTV, identified in Chapter 2, in the Huh7 cell line. It has confirmed the specific influence of LXRα in these results and discussed the implications of regulation by LXRα on the downstream gene expression of its metabolic targets, including SREBP-1c which has previously been implicated in PI induced lipodystrophy (Telenti *et al.*, 2002). This chapter has also hypothesised the role of multiple NRs, including LXRα, FXR, VDR, HNF4α and PPARα, controlling regulation of key downstream metabolic targets and discussed the specific role of LXRα in their regulation. Finally it has discussed the potential future clinical role for inhibition of LXRα as it has been shown that LXRα agonists have disruptive effects on a cascade of metabolic genes and downstream targets. Inhibition of LXRα has demonstrated that some of these downstream effects can be modified.

CHAPTER 4

THE IMPACT OF LIVER OXYSTEROL RECEPTOR ALPHA (LXRα) MODULATION BY RITONAVIR AND ATAZANAVIR ON TRANSPORTERS IN THE CHOLESTEROL METABOLISM PATHWAY

4.1 Introduction

LXRα is a master regulator of cholesterol homeostasis, bile acid metabolism, and modulates the transcription of many key genes involved in drug and lipid metabolism (Hui, 2003; Liang *et al.*, 2001; Osborne, 2000). LXRα has also been shown to have positive effects on glucose metabolism (Cha *et al.*, 2007; Kalaany *et al.*, 2006). In Chapters 2 and 3 of this thesis the impact of the PIs RTV and ATV on NR gene expression and the role played by LXRα in this was investigated and discussed. This Chapter will focus on the impact of RTV and ATV on key transporters in cholesterol, bile acid and glucose metabolism where LXRα has previously been implicated in regulation of gene expression.

Cholesterol occupies the spaces between the polar head-groups of the phospholipid bilayer of cell membranes where it reduces the fluidity of the membrane. It is also the precursor molecule for the synthesis of bile salts, vitamin D and steroid hormones (Charlton-Menys *et al.*, 2008). It can be derived from the diet or synthesised within the body by the liver and central nervous system (CNS). It can be lost from the body, as part of a daily faecal loss as fractions of bile salts and unabsorbed intestinal cholesterol, and in sebum (Charlton-Menys *et al.*, 2008; Levy *et al.*, 2007). Bile salts are detergents synthesised by the liver, from cholesterol, and released with bile into the duodenum. Bile salts emulsify fats via their hydrophilic polar carboxyl and hydroxyl groups, and their lipophilic hydrocarbon rings which interface with lipids (Charlton-Menys *et al.*, 2008). Cholesterol circulates as lipoproteins, principally as chylomicrons, LDL, VLDL and HDL (Charlton-Menys *et al.*, 2008; Lusis, 2000; Williams *et al.*, 2007).

4.1.1 Transporters Involved in Cholesterol Homeostasis, Bile Acid and Glucose Metabolism

Members of the ABC transporter family are transmembrane proteins which transport a wide variety of endogenous substrates and xenobiotics across extracellular and intracellular membranes. ABCB1 (P-glycoprotein, P-gp, multidrug resistance protein 1), ABCC1 and ABCC2 (the multidrug resistance associated proteins 1 and 2, respectively) have previously been implicated in modulating the levels of anti-retroviral drugs, including RTV and ATV, in cells and tissues (Ho *et al.*, 2005; Telenti *et al.*, 2002; Telenti *et al.*, 2008). ABCB1 has a critical role in drug absorption, biliary excretion, renal secretion and central nervous system (CNS) entry of a wide range of hydrophobic xenobiotics (Faber *et al.*, 2003; Ho *et al.*, 2005).

ABCB1 has previously been shown to be involved in the transport of both RTV and ATV, (Perloff *et al.*, 2005; Solon *et al.*, 2002). RTV and ATV have both been shown to be substrates for ABCB1 and ABCC1 (Janneh *et al.*) rendering these transporters suitable positive controls for this research.

ABCB1 and ABCC1 are mainly located in the plasma membrane of the epithelium and endothelium of the liver (Ho *et al.*, 2005; Pan *et al.*, 2007; Urquhart *et al.*, 2007). ABCC2 is a drug efflux transporter expressed in the liver and its primary function is the excretion of organic anions from Phase II drug metabolism reactions, e.g. glutathione, glucuronide and sulphate conjugates, from hepatocytes into bile. ABCC2 is involved in the transport of endogenous substrates including bilirubin and bile salts (Urquhart *et al.*, 2007). Sections 1.6.2 and 1.6.3 discussed the control of

ABCC2 expression by the NRs PXR, FXR and CAR. ABCC2 gene expression has been implicated in the metabolic disorder Dubin-Johnson Syndrome (Stefkova *et al.*, 2004).

FXR has also been implicated in the up-regulation of ABCB11 (the bile salt export pump, BSEP) (Eloranta *et al.*, 2005; Thomas *et al.*, 2008). Defects in ABCB11 have been linked to metabolic diseases including progressive familial intrahepatic cholestasis type 2 (Stefkova *et al.*, 2004). LXRα has been shown to be the counterbalance of FXR regulation (Berkenstam *et al.*, 2005; Edwards *et al.*, 2002; Kalaany *et al.*, 2006; Rader, 2007). LXRα has been implicated in the regulation of ABCA1 (Edwards *et al.*, 2002; Eloranta *et al.*, 2005), ABCG1, ABCG4, ABCG5 and ABCG8, key genes in cholesterol transport (Bradley *et al.*, 2005; Edwards *et al.*, 2002; Joseph *et al.*, 2003; Rader, 2007). Defects in the ABCA1 (cholesterol efflux regulatory protein, CERP) gene have been linked to the metabolic disorders Tangier disease and familial hypoapoproteinaemia (Stefkova *et al.*, 2004). Polymorphisms in ABCG5 and ABCG8 genes have been implicated in the metabolic disease sitosterolaemia (Stefkova *et al.*, 2004). ABCB2, is a half-ABC transporter, which functions as a peptide transporter involved in pumping degraded cytosolic peptides across the endoplasmic reticulum. It is a key player in bile acid transport. Mutations in ABCB2 have been associated with immune deficiencies including ankylosing spondylitis, coeliac disease and insulin dependent diabetes mellitus (Stefkova *et al.*, 2004).

GLUT4 (SLC2A4), a key gene involved in glucose metabolism, has been shown to be a direct target of LXRα (Mitro *et al.*, 2007; Zelcer *et al.*, 2006). Sections 2.5 and 2.6 of this thesis have already discussed the up-regulation of FXR and LXRα gene expression by RTV and the consequential effects on the downstream target SREBP-1c, where up-regulation by LXRα was seen to be the predominant consequence of incubation with RTV. SREBP-1c is a key player in the regulation of hepatic triglyceride and fatty acid synthesis (Zhang *et al.*, 2008) and a target of both LXRα and FXR, key players in cholesterol and bile acid homeostasis (Edwards *et al.*, 2002; Kalaany *et al.*, 2006). The different regulation of FXR and LXRα gene expression by RTV and ATV led to the hypothesis that cholesterol and bile acid metabolism and transport may also be disrupted through downstream regulation of transporter gene expression.

4.2 Aim

The aims of this chapter were to investigate the specific impact of the PIs RTV and ATV on the gene expression of transporters involved in cholesterol homeostasis and bile acid metabolism; and on the gene expression of the glucose transporter GLUT4; and to establish whether LXRα influences the gene expression of these transporters in the presence of RTV.

4.3 Materials and Methods

4.3.1 Materials

As previously described in Sections 2.3.1 and 3.3.1 above. Nunclon Surface 48 well plates were purchased from (Nunc A/S, Kamstrup, Denmark).

4.3.2 Cells and Cell Maintenance

4.3.2.1 Huh7 Cell Line

As previously described in Section 2.3.2.1 above.

4.3.2.2 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Toxicity Assays

The toxicity of RTV, ATV and 22-(S)-hydroxycholesterol was determined by the MTT assay at final concentrations as previously described in Section 2.3.2.2 of this thesis. Drugs were determined to be toxic where the cell viability after 120h was below 85% compared to the untreated cells.

4.3.2.3 Cell Treatment

Cells were seeded as previously described in Sections 2.3.2.3 and 3.3.2.3 above. The concentration–response experiments for RTV in the biological knockdown treated Huh7 cells were conducted at final concentrations of 0, 0.1, 1.0, and 10μM RTV following an initial 24h knockdown by siRNA, optimised as described in section 3.3.2.7 and set up as described previously in Section 3.3.2.8. The cells were incubated at 37°C and 5% CO_2 and were sampled at 24h.

4.3.2.4 RNA Isolation from Huh7 Cells

Isolation of RNA from untreated, RTV-treated, ATV-treated and RTV plus 22-(S)-hydroxycholesterol-treated Huh7 cells was as previously described in Section 2.3.2.4 of this thesis.

4.3.2.5 Production of cDNA

RNA was isolated as described above (2.3.2.4) and cDNA was synthesised in a total reaction volume of 50µl with the total RNA normalised to 2µg per 50µl reaction, as previously described in Section 2.3.2.5 of this thesis.

4.3.3 RT PCR – Gene Expression

Taqman Gene Expression inventoried assays for RT-PCR (Applied Biosystems Limited, Warrington, UK, (Table 4.1)) were used for ABCA1, ABCB1, ABCB2, ABCB11, ABCC1, ABCC2, ABCG1, ABCG4, ABCG5, ABCG8 and GLUT4 quantified against the housekeeping gene β2M. Relative expression of the transcripts against the expression of the housekeeping gene was performed, in duplicate for all assays as previously described in Section 2.3.2.9 above.

Table 4.1: Taqman Gene Expression Assays for RT PCR

Taqman Assay ID	Assay Gene
Hs01059118_m1	ABCA1
Hs00184491_m1	ABCB1
Hs00388682_m1	ABCB2
Hs00184824_m1	ABCB11
Hs00219905_m1	ABCC1
Hs00166123_m1	ABCC2
Hs01555189_m1	ABCG1
Hs00223446_m1	ABCG4
Hs03037375_m1	ABCG5
Hs02880035_m1	ABCG8
Hs99999907_m1	β2M
Hs00168966_m1	GLUT4

4.4 Statistical Analysis

Statistical analysis of all results was undertaken using StatsDirect Statistical software version 2.7.8 as previously described in Section 2.4 above.

4.5 Results

4.5.1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Toxicity Assays

Results for the MTT toxicity assays are shown in Section 2.5.1 of this thesis.

4.5.2 Quantification of RNA and cDNA

RNA was extracted from Huh7 cells, analysed and quantified, as previously described in Section 4.3.2.4 above. The RNA had an A_{260}/A_{280} ratio in the range of 1.85-2.01 (RTV samples), 1.94-2.15 (ATV samples), 1.92-2.08 (RTV + 22-(S)-hydroxycholesterol samples) and 1.85-1.97 (siRNA samples) indicating sufficient purity to proceed to cDNA construction for RT PCR. RNA samples were frozen at -80°C following cDNA construction.

The cDNA for all conditions had A_{260}/A_{280} ratios in the range of 1.98-2.13 indicating sufficient purity to proceed to construction of normalised (20ng/μl) cDNA working stocks. Master and working stock cDNA samples were frozen at -20°C until used in gene expression assays.

4.5.3 Gene Expression in Ritonavir, Atazanavir and 22(S)-Hydroxycholesterol/ Ritonavir Treated Huh 7 Cells

In the optimisation experiments in Sections 2.5.6 and 3.5.5, maximal up-regulation and down-regulation of gene expression by both RTV and ATV was observed after 24h incubation, so the same incubation period was utilised for the transporter gene expression assays.

Optimal conditions for siRNA knockdown of LXRα in Huh7 cells were determined to be 5.0μl Lipofectamine 2000 with 15nM final concentration LXRα siRNA in 6 well plates at the mRNA and protein levels over 24h (Section 3.5.5). Optimal conditions for siRNA knockdown of GAPDH (Positive control) and stable expression of non-targeting siRNA (negative control) were determined to be 5.0μl Lipofectamine 2000 with 20nM GAPDH siRNA and 5.0μl Lipofectamine 2000 with 20nM non-targeting siRNA in 6 well plates at the mRNA and protein levels over 24h (Section 3.5.4).

4.5.3.1 ABCA1

Significant up-regulation of ABCA1 gene expression by RTV was observed at concentrations of 0.1μM (1.9 fold increase compared to vehicle control, P=<0.0001), 1.0μM (2.0 fold increase compared to vehicle control, P=<0.0001) and 10μM (3.4 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 4.1 below. In the presence of 20μM 22-(S)-hydroxycholesterol, the statistically significant up-regulation of ABCA1 by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM was abolished and replaced with significant down-regulation of ABCA1 gene expression by 1.6 fold compared to vehicle controls at each RTV concentration (P=<0.0001 at concentrations of 0.1μM, 1.0μM and 10μM RTV plus 20μM 22-(S)-hydroxycholesterol), Figure 4.1 below. ABCA1 gene expression was not significantly altered in the presence of 0.1μM ATV, however, significant down-regulation of ABCA1 was observed at concentration of 1.0μM (1.9 fold decrease compared to vehicle control, P=<0.0001) and 10μM ATV (2.3 fold decrease compared to vehicle control, P=<0.0001), Figure 4.1 below. Significant up-regulation of ABCA1 gene expression by RTV was observed in the mock

transfections (5.0μl Lipofectamine 2000/0nM siRNA) at concentrations of 0.1μM (1.9 fold increase compared to vehicle control, P=<0.0001), 1.0μM (2.0 fold increase compared to vehicle control, P=<0.0001) and 10μM (2.7 fold increase compared to vehicle control, P=<0.0001), Figure 4.1 below. The expression of ABCA1 in siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 4.1 below. The statistically significant up-regulation of ABCA1 gene expression by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was abolished and replaced with significant down-regulation of ABCA1 gene expression by 2.7 fold compared to vehicle controls at 0.1μM RTV (P=<0.001), by 1.9 fold compared to vehicle controls at 1.0μM (P=<0.0001) and by 1.8 fold at 10μM (P=<0.001), in the siRNA LXRα knockdown samples, Figure 4.1 below.

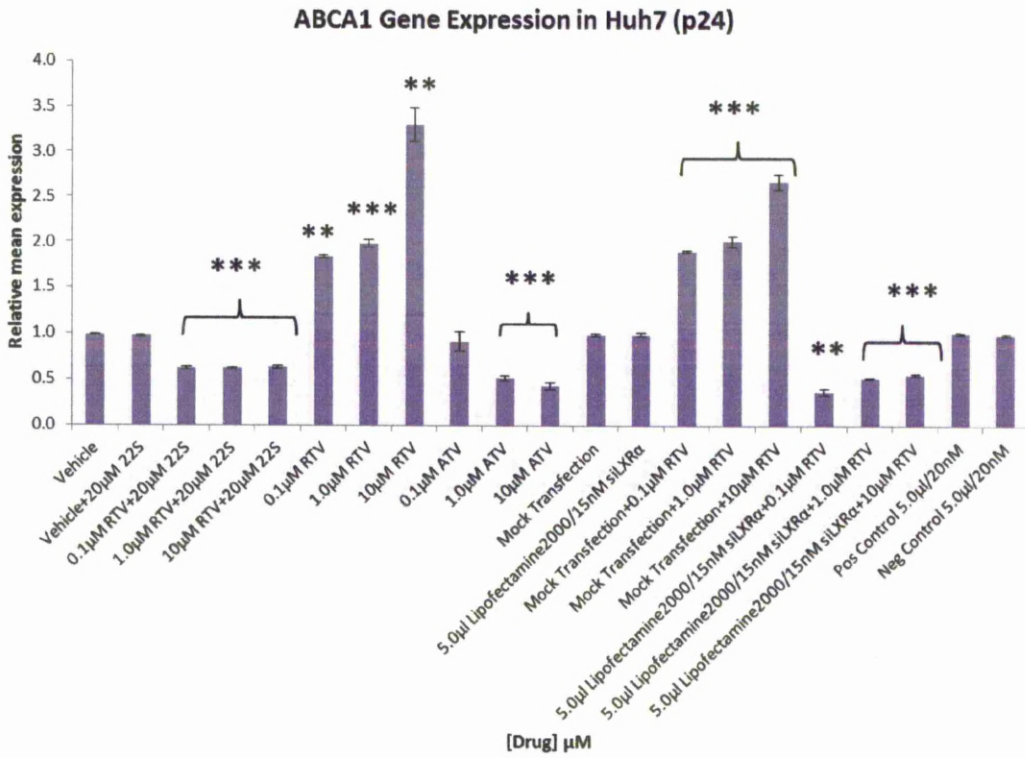


Figure 4.1: ABCA1 Gene expression assays in Huh7 cells (passage no. = 24) incubated with ritonavir (RTV), RTV plus 20μM 22(S)-Hydroxycholesterol, RTV in Mock Transfection cells, RTV in 24h siRNA knockdown cells and atazanavir (ATV) for 24h: n=4 for all assays. *** denotes P=<0.0001, ** denotes P=0.001* denotes P=<0.01.

4.5.3.2 ABCB1, ABCB2 and ABCB11

ABCB1 gene expression was significantly down-regulated by RTV at 0.1μM (8.2 fold decrease compared to vehicle control, P=<0.0001), 1.0μM (47.2 fold decrease compared to vehicle control, P=<0.0001) and 10μM (9.3 fold decrease compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 4.2 below. The statistically significant down-regulation of ABCB1 observed at all RTV concentrations was abolished and replaced with significant up-regulation of ABCB1 gene expression in the presence of 20μM 22-(S)-hydroxycholesterol, at 0.1μM RTV (1.7 fold increase compared to vehicle controls P=<0.0001), 1.0μM (1.8 fold increase compared to

vehicle controls P=0.0034), and 10μM (1.6 fold increase compared to vehicle controls P=0.0018), Figure 4.2 below. ABCB1 gene expression was down-regulated by ATV at all concentrations, at 0.1μM there was a significant 1.6 fold decrease compared to vehicle controls, (P=0.0046), but at 1.0μM there was only a 1.1 fold decrease compared to vehicle controls, which was not statistically significant (P=0.5182) and at 10μM ATV there was a significant 1.3 fold decrease compared to vehicle controls, (P=0.0103) in Huh7 cells (n=4), Figure 4.2 below. Highly significant down-regulation of ABCB1 gene expression by RTV was observed in the mock transfections (5.0μl Lipofectamine 2000/0nM siRNA) at RTV concentrations of 0.1μM (2.3 fold decrease compared to vehicle control, P=<0.0001), 1.0μM (4.5 fold decrease compared to vehicle control, P=<0.0001) and 10μM (8.4 fold decrease compared to vehicle control, P=<0.0001), Figure 4.2 below. The expression of ABCB1 in siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 4.2 below. The statistically significant down-regulation of ABCB1 gene expression by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was abolished and replaced with a highly significant up-regulation of ABCB1 gene expression by 1.9 fold compared to vehicle controls at 0.1μM RTV (P=<0.0001), by 1.8 fold compared to vehicle controls at 1.0μM (P=<0.0001) and by 1.6 fold at 10μM (P=<0.0008), in the siRNA LXRα knockdown samples, Figure 4.2 below.

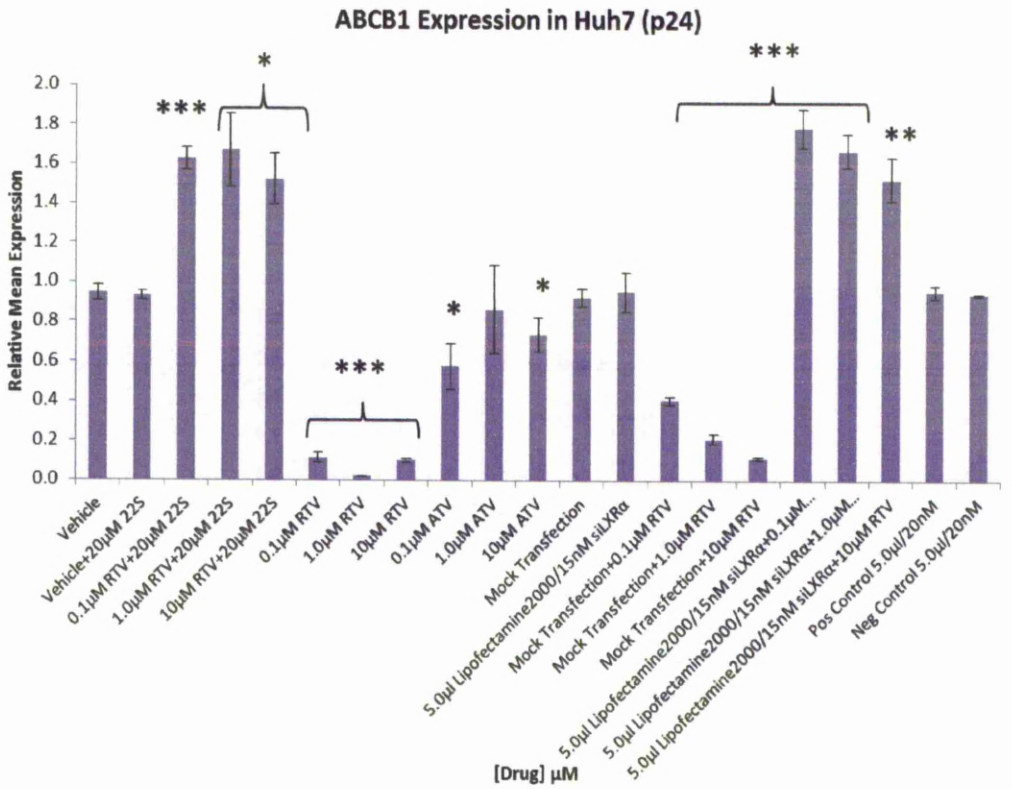


Figure 4.2: ABCB1 Gene expression assays in Huh7 cells (passage no. = 24) incubated with ritonavir (RTV), RTV plus 20μM 22(S)-Hydroxycholesterol, RTV in Mock Transfection cells, RTV in 24h siRNA knockdown cells and atazanavir (ATV) for 24h: n=4 for all assays. *** denotes P=<0.0001, ** denotes P=0.001* denotes P=<0.01.

A highly significant up-regulation of ABCB2 gene expression by RTV was observed at concentration of 0.1μM (1.9 fold increase compared to vehicle control, P=<0.0001), however, at higher RTV concentrations a down-regulation of ABCB2 gene expression was observed. At 1.0μM RTV a statistically significant 1.1 fold decrease compared to vehicle controls, P=0.0371 was observed and at 10μM RTV a highly significant 2 fold decrease in ABCB2 expression compared to vehicle controls was seen, (P=0.0002) in Huh7 cells (n=4), Figure 4.3 (A) below. In the presence of 20μM 22-(S)-hydroxycholesterol, the statistically significant up-regulation of ABCB2 by RTV observed at 0.1μM was abolished and replaced with significant

down-regulation of ABCB2 gene expression by 1.1 fold compared to vehicle controls (P=0.0225). At 1.0μM RTV plus 20μM 22-(S)-hydroxycholesterol the regulation was unaltered from the same concentration RTV alone assay, down-regulation was 1.1 fold compared to vehicle controls with a significance at P=0.0404, Figure 4.3 (A) below. At 10μM RTV plus 20μM 22-(S)-hydroxycholesterol there was no significant difference in ABCB2 gene expression compared to vehicle controls, Figure 4.3 (A) below. ABCB2 gene expression was significantly down-regulated by ATV at all concentrations, at 0.1μM there was a significant 8 fold decrease compared to vehicle controls, (P=<0.0001), at 1.0μM there was a 2.3 fold decrease compared to vehicle controls, (P=<0.0001) and at 10μM ATV there was a significant 1.3 fold decrease compared to vehicle controls, (P=0.0053) in Huh7 cells (n=4), Figure 4.3 (A) below. Significant up-regulation of ABCB2 gene expression by RTV was observed in the mock transfections (5.0μl Lipofectamine 2000/0nM siRNA) at 0.1μM (1.8 fold increase compared to vehicle control, P=0.0017), furthermore at 1.0μM RTV (1.1 fold decrease compared to vehicle control, P=<0.0001) and 10μM a 2.1 fold decrease in ABCB2 expression compared to vehicle control was observed, (P=<0.0001), Figure 4.3 (A) below. The expression of ABCB2 in siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 4.3 (A) below. The statistically significant up-regulation and down-regulation of ABCB2 gene expression by RTV observed at concentrations of 0.1μM and 1.0μM RTV with and without mock transfection was abolished in the siRNA LXRα knockdown samples, at 10μM RTV a significant down-regulation of ABCB2 gene expression by 1.3 fold compared to

vehicle controls (P=0.006), was observed in the siRNA LXRα knockdown samples, Figure 4.3 (A) below.

Significant up-regulation of ABCB11 gene expression by RTV was observed at 0.1μM (2.9 fold increase compared to vehicle control, P=<0.0001), however, highly significant down regulation of ABCB11 gene expression was observed at concentrations of 1.0μM RTV (1.9 fold decrease compared to vehicle control, P=<0.0001) and 10μM (2.9 fold decrease compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 4.3 (B) below. In the presence of LXRα antagonist, 20μM 22-(S)-hydroxycholesterol, the statistically significant up-regulation and down-regulation of ABCB11 expression by RTV was reversed. At 0.1μM RTV in the presence of the antagonist ABCB11 gene expression was down-regulated 1.2 fold compared to vehicle controls (P=<0.0001), at 1.0μM and 10μM RTV, in the presence of the antagonist, ABCB11 expression was significantly up-regulated by 1.2 and 1.3 fold respectively compared to vehicle controls (P=<0.0001 at both 1.0μM and 10μM RTV plus 20μM 22-(S)-hydroxycholesterol), Figure 4.3 (B) below. ABCB11 gene expression was significantly up-regulated in the presence of 0.1μM (1.3 fold increase compared to vehicle controls, P=0.0002) and 1.0μM ATV (1.2 fold increase compared to vehicle controls, P=0.0002), however, significant down-regulation of ABCB11 was observed at concentration of 10μM ATV (1.4 fold decrease compared to vehicle control, P=0.0011), Figure 4.3 (B) below. Significant up-regulation of ABCB11 gene expression by RTV was observed in the mock transfections (5.0μl Lipofectamine 2000/0nM siRNA) at 0.1μM RTV (2.8 fold increase compared to vehicle control, P=0.0002). Significant down-regulation of

ABCB11 was observed in the mock transfections at 1.0μM RTV (2.3 fold decrease compared to vehicle control, P=<0.0001) and 10μM (2.4 fold decrease compared to vehicle control, P=<0.0001), Figure 4.3 (B) below. The expression of ABCB11 in siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 4.3 (B) below. The statistically significant up-regulation of ABCB11 gene expression by RTV observed at 0.1μM with and without mock transfection was abolished and replaced with significant down-regulation of ABCB11 gene expression by 1.2 fold compared to vehicle controls, (P=0.0019). The down-regulation observed at 1.0μM and 10μM RTV with and without mock transfection was still evident although at lesser levels with 1.3 fold decreases compared to vehicle controls at both 1.0μM RTV (P=0.0065) and 10μM RTV (P=0.0043), in the siRNA LXRα knockdown samples, Figure 4.3 (B) below.

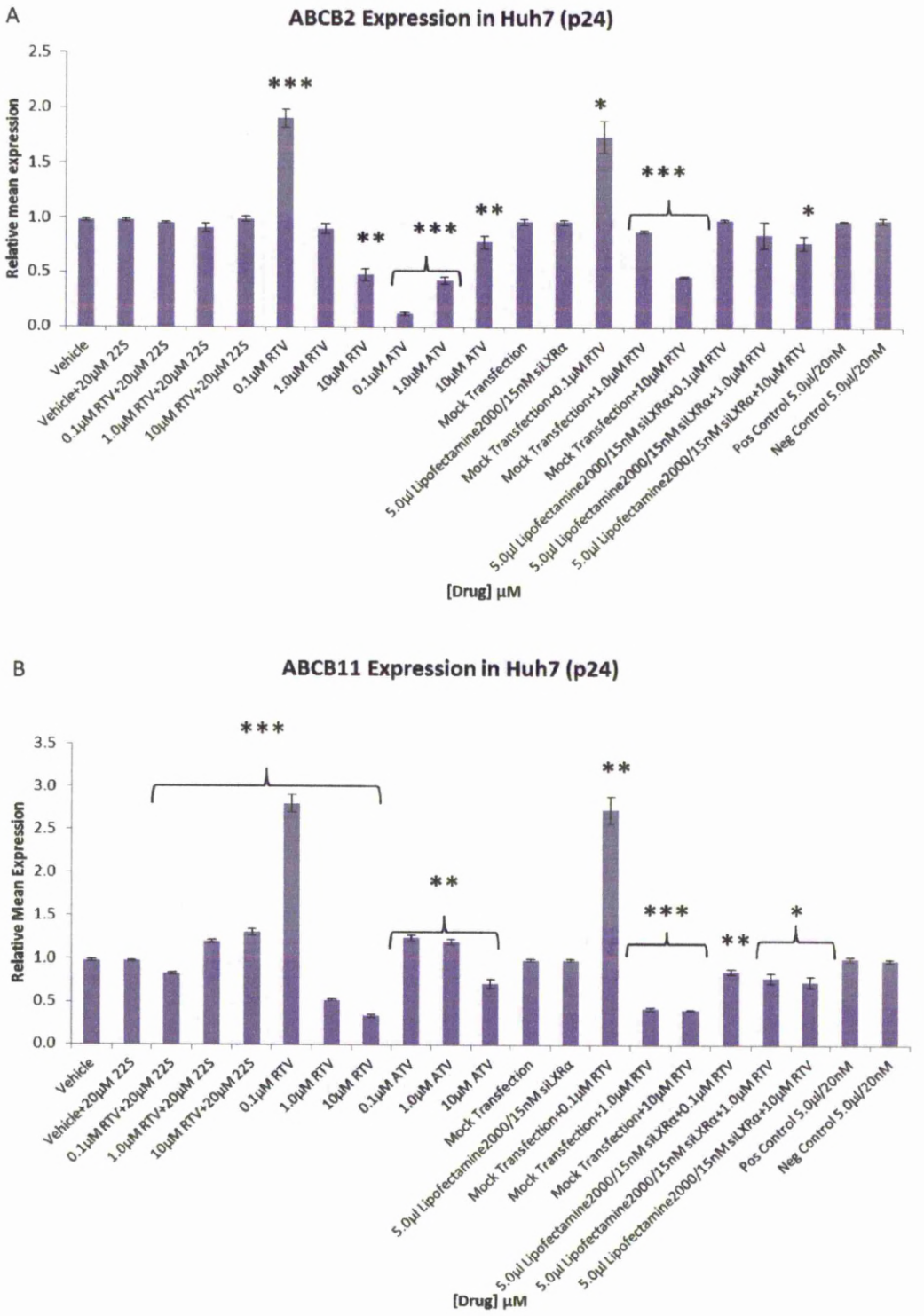


Figure 4.3: Gene expression assays in Huh7 cells (passage no.=24) incubated with ritonavir (RTV), RTV plus 20μM 22(S)-Hydroxycholesterol, RTV in Mock Transfection cells, RTV in 24h siRNA knockdown cells and atazanavir (ATV) for 24h: A= ABCB2; and B= ABCB11, n=4 for all assays. *** denotes P=<0.0001, ** denotes P=<0.001 * denotes P=<0.01.

4.5.3.3 ABCC1 and ABCC2

Highly significant down-regulation of ABCC1 gene expression by RTV was observed at concentrations of 0.1μM (7 fold decrease compared to vehicle control, P=<0.0001), 1.0μM (9 fold decrease compared to vehicle control, P=<0.0001) and 10μM (4.5 fold decrease compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 4.4 (A) below. In the presence of 20μM 22-(S)-hydroxycholesterol, the statistically significant down-regulation of ABCC1 by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV was abolished at 0.1μM RTV plus 20μM 22-(S)-hydroxycholesterol and had diminished down-regulation of ABCC1 expression at 1.0μM (1.9 fold decrease compared to vehicle controls, P=<0.0001) and 10μM (2.3 fold decrease compared to vehicle controls, P=<0.0001), Figure 4.4 (A) below. ABCC1 gene expression was also significantly down-regulated in the presence of all concentrations of ATV. At 0.1μM ATV ABCC1 gene expression was down-regulated 12.7 fold compared to vehicle controls, (P=<0.00001), at 1.0μM ATV there was a 3.1 fold decrease compared to vehicle controls, (P=<0.0001) and at 10μM ATV ABCC1 gene expression was significantly down-regulated by 3.5 fold compared to vehicle controls, (P=<0.0001), Figure 4.3 (A) below. Significant down-regulation of ABCC1 gene expression by RTV was observed in the mock transfections (5.0μl Lipofectamine 2000/0nM siRNA) at concentrations of 0.1μM (6.3 fold decrease compared to vehicle control, P=<0.0001), 1.0μM (9.5 fold decrease compared to vehicle control, P=<0.0001) and 10μM (4.7 fold decrease compared to vehicle control, P=<0.0001), Figure 4.4 (A) below. The expression of ABCC1 in siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 4.4 (A) below. The statistically

significant down-regulation of ABCC1 gene expression by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was diminished but still significant down-regulation of ABCC1 by 1.1 fold compared to vehicle controls at 0.1μM RTV (P=<0.0001), by 2.1 fold compared to vehicle controls at 1.0μM (P=0.0008) and by 2.4 fold at 10μM (P=<0.0001), in the siRNA LXRα knockdown samples, Figure 4.4 (A) below.

Significant up-regulation of ABCC2 gene expression by RTV was observed at concentrations of 0.1μM (4.6 fold increase compared to vehicle control, P=<0.0001), and 1.0μM (2.1 fold increase compared to vehicle control, P=<0.0001), however, significant down-regulation of ABCC2 expression was observed at 10μM RTV (1.2 fold decrease compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 4.4 (B) below. In the presence of 20μM 22-(S)-hydroxycholesterol, the statistically significant up-regulation of ABCC2 by RTV observed at 0.1μM, 1.0μM and 10μM was diminished to 1.1. fold, 1.3 fold and 1.1 fold increases in expression respectively, (P=<0.0001 at 0.1μM and 1.0μM and P=0.0035 at 10μM), Figure 4.4 (B) below. ABCC2 gene expression was significantly down-regulated in the presence of 0.1μM ATV (4.1 fold decrease compared to vehicle controls, P=<0.0001), and at 1.0μM ATV (1.8 fold decrease compared to vehicle controls, P=<0.0001), however, up-regulation of ABCC2 was observed at concentration of 10μM ATV (1.02 fold increase compared to vehicle control, P=0.0428), Figure 4.4 (B) below. Significant up-regulation of ABCC2 gene expression by RTV was observed in the mock transfections (5.0μl Lipofectamine 2000/0nM siRNA) at concentrations of 0.1μM (4.6 fold increase compared to vehicle control, P=<0.0001) and 1.0μM (2.6 fold

increase compared to vehicle control, P=<0.0001), however, at 10μM there were no significant differences from basal expression levels, Figure 4.4 (B) below. The expression of ABCC2 in siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 4.3 (B) below. The statistically significant up-regulation of ABCC2 gene expression by RTV observed at concentrations of 0.1μM and 1.0μM RTV, together with the significant down-regulation evidenced at 10μM RTV with and without mock transfection was again diminished at all RTV concentrations, ABCC2 gene expression was up-regulated by 1.1 fold compared to vehicle controls at 0.1μM RTV (P=0.0002), by 1.2 fold compared to vehicle controls at 1.0μM (P=0.0041) and down-regulated by 1.1 fold at 10μM (P=<0.0003), in the siRNA LXRα knockdown samples, Figure 4.4 (B) below.

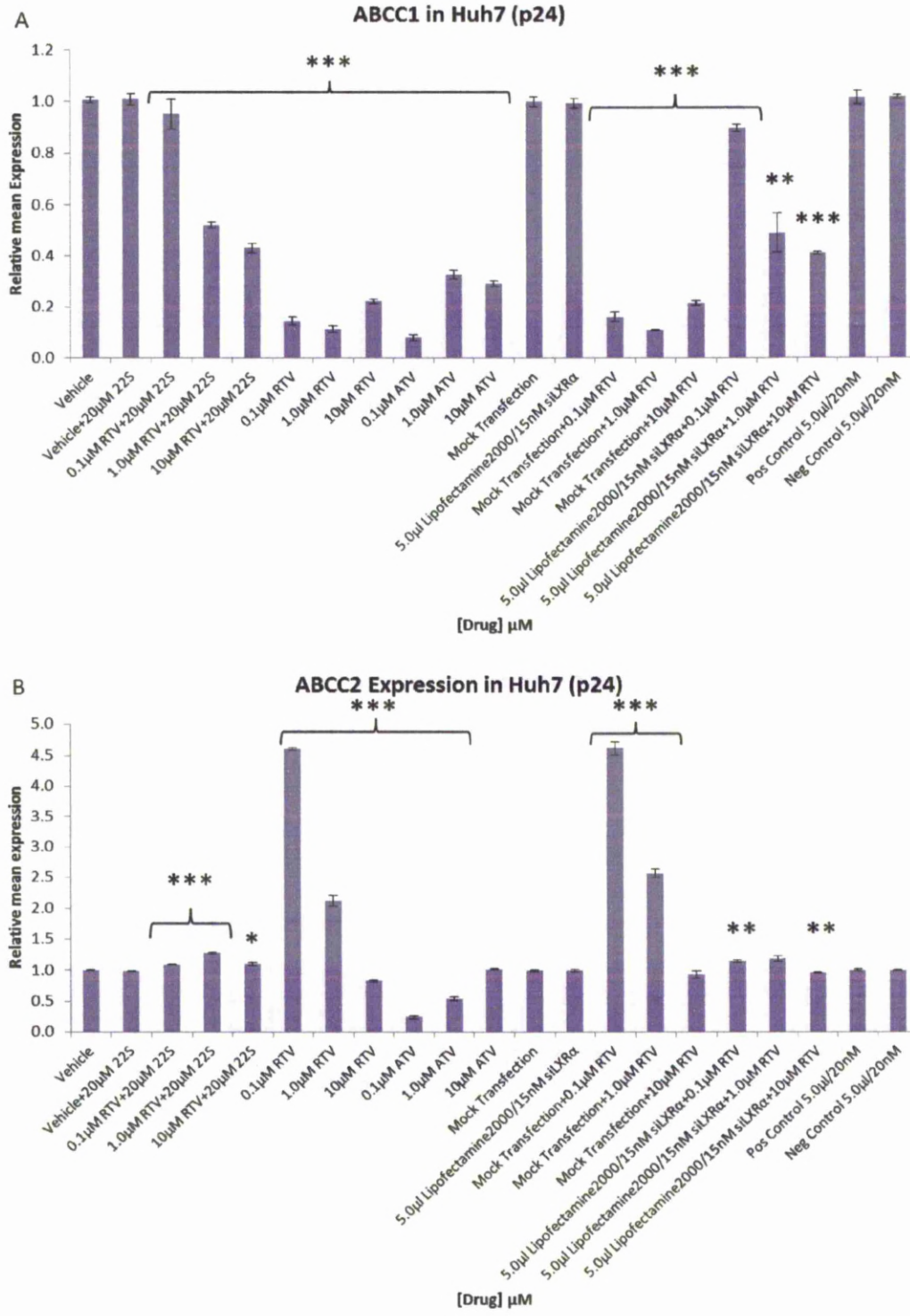


Figure 4.4: Gene expression assays in Huh7 cells (passage no.=24) incubated with RTV, RTV plus 20μM 22(S)-Hydroxycholesterol, RTV in Mock Transfection cells, RTV in 24h siRNA knockdown cells and ATV for 24h: A= ABCC1; and B= ABCC2, n=4 for all assays. *** denotes P=<0.0001, ** denotes P=<0.001 * denotes P=<0.01.

4.5.3.4 ABCG1, ABCG4, ABCG5 and ABCG8

Significant up-regulation of ABCG1 gene expression by RTV was observed at concentrations of 0.1μM (3.4 fold increase compared to vehicle control, P=0.0024), 1.0μM (3.9 fold increase compared to vehicle control, P=<0.0001) and 10μM (4.5 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 4.5 (A) below. In the presence of 20μM 22-(S)-hydroxycholesterol, the statistically significant up-regulation of ABCG1 by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM was diminished to 1.5 fold increase compared to vehicle controls at 0.1μM RTV (P=0.0008) at 1.0μM to a 2.5 fold increase compared to vehicle controls (P=<0.0001) and at 10μM RTV plus 20μM 22-(S)-hydroxycholesterol to a 3.1 fold increase compared to vehicle controls (P=0.0012), Figure 4.5 (A) below. ABCG1 gene expression was significantly down-regulated in the presence of all ATV concentrations, at 0.1μM ATV there was a 2.4 fold decrease in ABCG1 expression compared to vehicle controls, (P=0.0007), and at 1.0μM ATV a 4.5 fold decrease compared to vehicle controls was evident, (P=<0.0001), and at 10μM ATV a 4.1 fold decrease in expression compared to vehicle controls was observed, P=<0.0001), Figure 4.5 (A) below. Significant up-regulation of ABCA1 gene expression by RTV was observed in the mock transfections (5.0μl Lipofectamine 2000/0nM siRNA) at concentrations of 0.1μM (2.4 fold increase compared to vehicle control, P=0.0001), 1.0μM (3.9 fold increase compared to vehicle control, P=<0.0001) and 10μM (5.7 fold increase compared to vehicle control, P=<0.0001), Figure 4.5 (A) below. The expression of ABCG1 in siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 4.5 (A) below. The statistically significant up-regulation of ABCG1 gene

expression by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was diminished to a 1.6 fold increase compared to vehicle controls at 0.1μM RTV (P=0.0016), to a 1.6 fold increase compared to vehicle controls at 1.0μM (P=0.0059) and to a 1.9 fold increase at 10μM (P=<0.001), in the siRNA LXRα knockdown samples, Figure 4.5 (A) below.

Significant up-regulation of ABCG4 gene expression by RTV was observed at concentrations of 0.1μM (1.6 fold increase compared to vehicle control, P=<0.0001), 1.0μM (3.3 fold increase compared to vehicle control, P=<0.0001) and 10μM (6.9 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 4.5 (B) below. In the presence of 20μM 22-(S)-hydroxycholesterol, the statistically significant up-regulation of ABCG4 by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM was diminished to a 1.1 fold increase compared to vehicle controls at 0.1μM RTV (P=<0.0001), and was abolished and replaced with a significant down-regulation of ABCG4 gene expression at 1.0μM (1.1 fold decrease compared to vehicle controls, (P=<0.0001)) and 10μM (1.1 fold decrease compared to vehicle controls, (P=<0.0001)) RTV plus 20μM 22-(S)-hydroxycholesterol, Figure 4.5 (B) below. ABCG4 gene expression was significantly altered at all ATV concentrations, in the presence of 0.1μM ATV a 1.5 fold increase in gene expression compared to vehicle controls was observed, (P=<0.0001), however, significant down-regulation of ABCG4 expression was observed at concentrations of 1.0μM (1.1 fold decrease compared to vehicle control, P=<0.0001) and 10μM ATV (3.1 fold decrease compared to vehicle control, P=<0.0001), Figure 4.5 (B) below. Significant up-regulation of ABCG4 gene expression by RTV was observed in the mock

transfections (5.0μl Lipofectamine 2000/0nM siRNA) at concentrations of 0.1μM (1.8 fold increase compared to vehicle control, P=0.0005), 1.0μM (3.6 fold increase compared to vehicle control, P=<0.0001) and 10μM (6.8 fold increase compared to vehicle control, P=<0.0001), Figure 4.5 (B) below. The expression of ABCG4 in siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 4.5 (B) below. The statistically significant up-regulation of ABCG4 gene expression by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was abolished and replaced with significant down-regulation of ABCG4 gene expression by 1.4 fold compared to vehicle controls at 0.1μM RTV (P=0.0005), by 1.3 fold compared to vehicle controls at 1.0μM (P=0.0003) and by 1.1 fold at 10μM (P=0.0305), in the siRNA LXRα knockdown samples, Figure 4.5 (B) below.

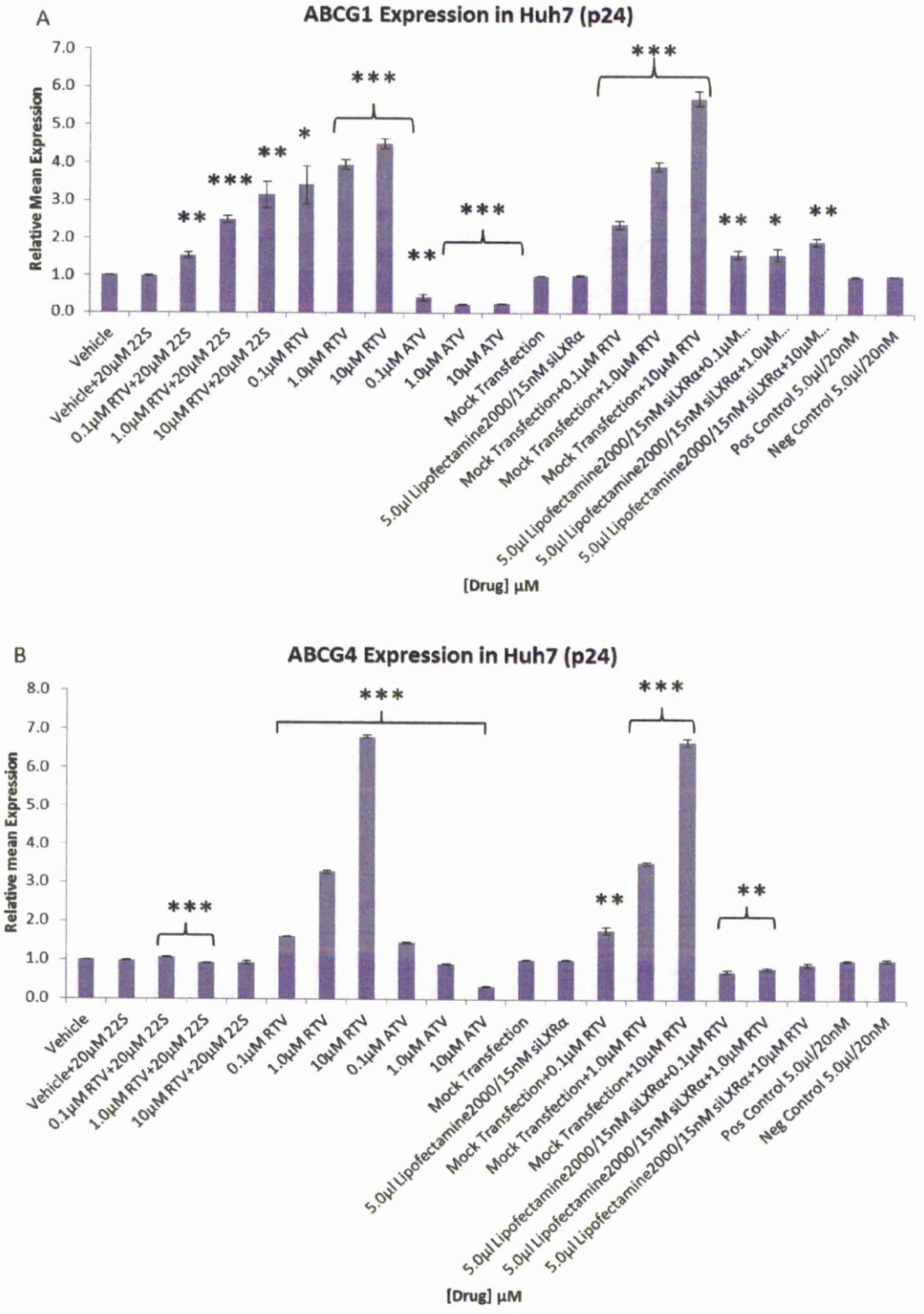


Figure 4.5: Gene expression assays in Huh7 cells (passage no.=24) incubated with RTV, RTV plus 20μM 22(S)-Hydroxycholesterol, RTV in Mock Transfection cells, RTV in 24h siRNA knockdown cells and ATV for 24h: A= ABCG1; and B= ABCG4, n=4 for all assays. *** denotes P=<0.0001, ** denotes P=<0.001 * denotes P=<0.01.

Significant up-regulation of ABCG5 gene expression by RTV was observed at concentrations of 0.1μM (2.2 fold increase compared to vehicle control, P=0.0003), 1.0μM (4.8 fold increase compared to vehicle control, P=0.0002) and 10μM (8.5 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 4.6 (A) below. In the presence of 20μM 22-(S)-hydroxycholesterol, the statistically significant up-regulation of ABCG5 by RTV observed at concentrations of 0.1μM and 10μM was diminished and at 1.0μM was abolished. ABCG5 gene expression at 0.1μM and 10μM RTV in the presence of 20μM 22-(S)-hydroxycholesterol was diminished to a 1.1 fold increase compared to vehicle controls at 0.1μM (P=0.0009) and at 10μM a 1.2 fold increase compared to vehicle controls was observed, (P=0.0242), Figure 4.6 (A) below. ABCG5 gene expression was significantly down-regulated at all ATV concentrations, in the presence of 0.1μM ATV a 3.5 fold decrease in gene expression compared to vehicle controls was observed, (P=<0.0001), at 1.0μM a 14.4 fold decrease in ABSG5 expression compared to vehicle control was observed, (P=<0.0001) and at 10μM ATV a 26.8 fold decrease compared to vehicle control was seen, (P=<0.0001), Figure 4.6 (A) below. Significant up-regulation of ABCG5 gene expression by RTV was observed in the mock transfections (5.0μl Lipofectamine 2000/0nM siRNA) at concentrations of 0.1μM (2.1 fold increase compared to vehicle control, P=<0.0001), 1.0μM (4.3 fold increase compared to vehicle control, P=0.0005) and 10μM (8.3 fold increase compared to vehicle control, P=<0.0001), Figure 4.6 (A) below. The expression of ABCG5 in siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 4.6 (A) below. The statistically significant up-regulation of ABCG5 gene expression by RTV observed at

concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was abolished at 0.1μM and diminished at 1.0μM and 10μM to a 1.1 fold increase compared to vehicle controls at 1.0μM (P=0.0107) and to a 1.3 fold increase at 10μM (P=<0.0001), in the siRNA LXRα knockdown samples, Figure 4.6 (A) below.

Significant up-regulation of ABCG8 gene expression by RTV was observed at concentrations of 0.1μM (2 fold increase compared to vehicle control, P=<0.0001), 1.0μM (3.7 fold increase compared to vehicle control, P=<0.0001) and 10μM (7.1 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 4.6 (B) below. In the presence of 20μM 22-(S)-hydroxycholesterol, the statistically significant up-regulation of ABCG8 by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM was diminished to a 1.9 fold increase in ABCG8 gene expression compared to vehicle controls at both 0.1μM and 1.0μM RTV concentrations (P=<0.0001 for both) and to a 1.7 fold increase compared to vehicle controls at 10μM RTV plus 20μM 22-(S)-hydroxycholesterol, (P=<0.0001), Figure 4.6 (B) below. ABCG8 gene expression was significantly up-regulated in the presence of 0.1μM ATV a 1.2 fold increase compared to vehicle controls (P=0.0012), however, significant down-regulation of ABCG8 was observed at concentration of 1.0μM (1.1 fold decrease compared to vehicle control, P=0.0004) and 10μM ATV (1.1 fold decrease compared to vehicle control, P=0.0104), Figure 4.6 (B) below. Significant up-regulation of ABCG8 gene expression by RTV was observed in the mock transfections (5.0μl Lipofectamine 2000/0nM siRNA) at concentrations of 0.1μM (2 fold increase compared to vehicle control, P=<0.0001), 1.0μM (4.2 fold increase compared to vehicle control, P=0.0003) and 10μM (9.5 fold increase compared to

vehicle control, P=<0.0001), Figure 4.6 (B) below. The expression of ABCG8 in siRNA LXRα knockdown cells without RTV was not significantly altered from basal expression in vehicle controls, Figure 4.6 (B) below. The statistically significant up-regulation of ABCG8 gene expression by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM RTV with and without mock transfection was abolished and replaced with significant down-regulation of ABCG8 gene expression by 1.1 fold compared to vehicle controls at 0.1μM RTV (P=0.0099), and diminished to a 1.1 fold increase compared to vehicle controls at 1.0μM (P=0.0287) and to a 1.3 fold increase at 10μM (P=0.0002), in the siRNA LXRα knockdown samples, Figure 4.6 (B) below.

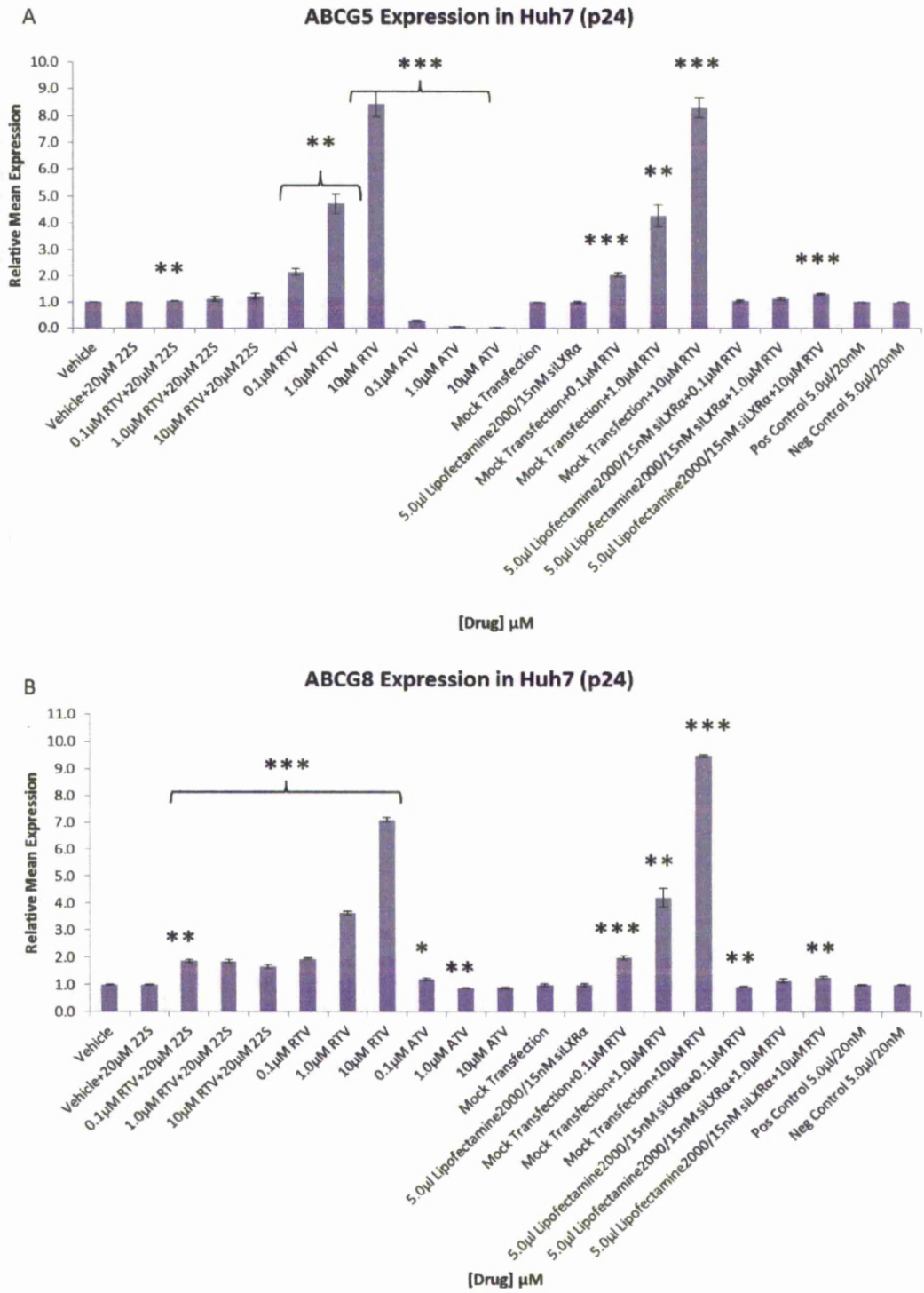


Figure 4.6: Gene expression assays in Huh7 cells (passage no.=24) incubated with RTV, RTV plus 20μM 22(S)-Hydroxycholesterol, RTV in Mock Transfection cells, RTV in 24h siRNA knockdown cells and ATV for 24h: A= ABCG5; and B= ABCG8, n=4 for all assays. *** denotes P=<0.0001, ** denotes P=<0.001 * denotes P=<0.01.

4.5.3.5 GLUT4

Significant up-regulation of GLUT4 gene expression by RTV was observed at concentrations of 0.1μM (1.6 fold increase compared to vehicle control, P=0.0022), 1.0μM (1.6 fold increase compared to vehicle control, P=<0.0001) and 10μM (2.5 fold increase compared to vehicle control, P=<0.0001) in Huh7 cells (n=4), Figure 4.7 below. In the presence of 20μM 22-(S)-hydroxycholesterol, the statistically significant up-regulation of GLUT4 by RTV observed at concentrations of 0.1μM, 1.0μM and 10μM was diminished at 0.1μM and 10μM, and abolished at 1.0μM. At 0.1μM RTV in the presence of 20μM 22-(S)-hydroxycholesterol GLUT4 gene expression was diminished to a 1.03 fold increase compared to vehicle controls, (P=0.0243) and at 10μM RTV plus 20μM 22-(S)-hydroxycholesterol) GLUT4 gene expression diminished to a 1.2 fold increase compared to vehicle controls, (P=<0.0001) Figure 4.7 below. GLUT4 gene expression was significantly down-regulated in the presence of all concentrations of ATV. At 0.1μM ATV, an 8 fold decrease in gene expression was observed compared to vehicle controls, (P=<0.0001) significant down-regulation of GLUT4 was also observed at 1.0μM ATV a 4 fold decrease compared to vehicle controls, (P=<0.0001) and at 10μM ATV a 4.8 fold decrease compared to vehicle controls, (P=<0.0001), Figure 4.7 below. Significant up-regulation of GLUT4 gene expression by RTV was observed in the mock transfections (5.0μl Lipofectamine 2000/0nM siRNA) at concentrations of 0.1μM (2.1 fold increase compared to vehicle control, P=<0.0001), 1.0μM (2 fold increase compared to vehicle control, P=<0.0001) and 10μM (2.4 fold increase compared to vehicle control, P=<0.0001), Figure 4.7 below. The expression of GLUT4 in siRNA LXRα knockdown cells without RTV was not significantly altered from basal

expression in vehicle controls, Figure 4.6 below. The statistically significant up-regulation of GLUT4 gene expression by RTV observed at concentrations of 0.1μM and 1.0μM with and without mock transfection was abolished and replaced with down-regulation of GLUT4 gene expression by 1.1 fold decrease compared to vehicle controls at 0.1μM RTV (P=0.0334), and by 1.1 fold decrease compared to vehicle controls at 1.0μM (P=0.0586, not significant). At 10μM RTV in the siRNA LXRα knockdown samples, a diminished significant up-regulation of GLUT4 gene expression was observed at a 1.1 fold increase compared to vehicle controls, (P=0.0006), Figure 4.7 below.

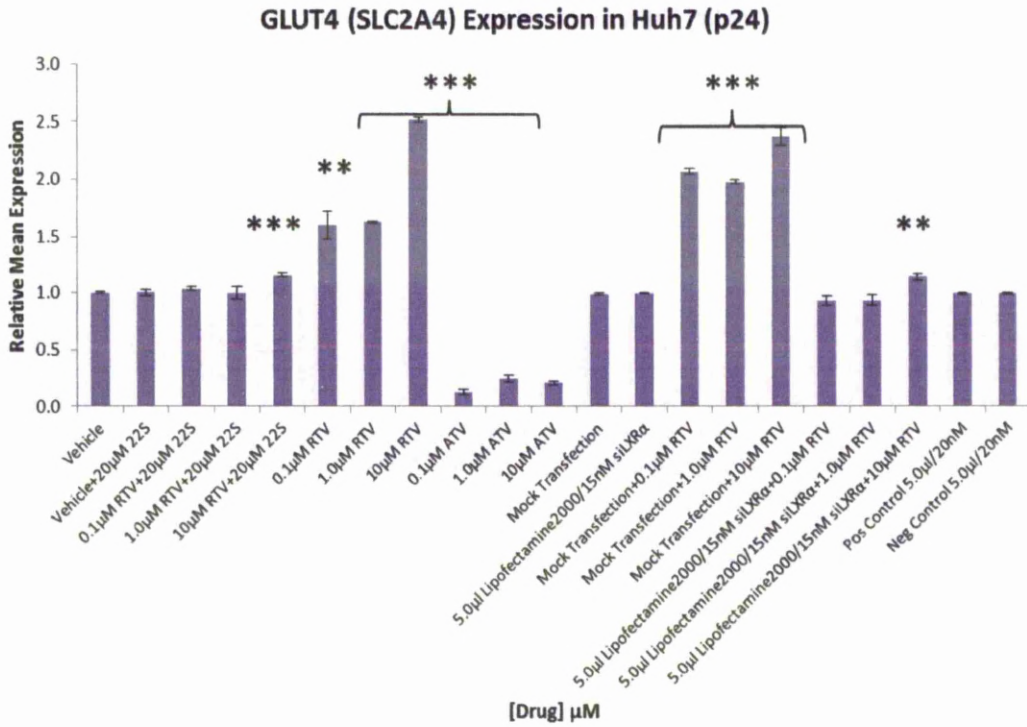


Figure 4.7: GLUT4 Gene expression assays in Huh7 cells (passage no.=24) incubated with RTV, RTV plus 20μM 22(S)-Hydroxycholesterol, RTV in Mock Transfection cells, RTV in 24h siRNA knockdown cells and ATV for 24h, n=4 for all assays. *** denotes P=<0.0001, ** denotes P=<0.001 * denotes P=<0.01

4.6 Discussion

Chapter 2 and Chapter 3 of this thesis have demonstrated that LXRα is involved in the differing target gene expression profiles associated with the PIs RTV and ATV. This thesis has also demonstrated that antagonism of LXRα and biological knockdown by siRNA can be used to demonstrate the role of LXRα in the response to RTV. The endogenous role of LXRα is as a master regulator of cholesterol homeostasis in the liver and macrophages (Gronemeyer *et al.*, 2004a; Kalaany *et al.*, 2006; Pascussi *et al.*, 2008). It facilitates the processing and storage of cholesterol by up-regulating the cytochrome P450 7A1 enzyme (CYP7A1) and has been implicated in the increased expression of the ATP binding cassette proteins ABCA1, ABCG1, ABCG5 and ABCG8, key players in cholesterol transport and metabolism (Eloranta *et al.*, 2005; Pascussi *et al.*, 2008; Tirona *et al.*, 2005). FXR, provides the counterbalance of hepatic LXRα action. It is activated by bile acid accumulation resulting from the conversion of cholesterol to bile acids, itself regulated by LXRα. Inhibition of bile acid synthesis is achieved by FXR down regulating CYP7A1, through indirect signalling involving the increased expression of the small heterodimer partner-1 (SHP-1)-liver receptor homologue-1 (LRH-1) pathway. SHP-1 represses CYP7A1 expression by binding to promoter sequences and preventing further RE binding of activated LXRα (Eloranta *et al.*, 2005).

Transcriptional activity of LXRα is induced by elevated levels of naturally occurring oxysterols and 6α-hydroxy bile acids (Eloranta *et al.*, 2005; Francis *et al.*, 2003). LXRα is also abundant in adipose tissue, where its activation has been shown to increase basal glucose uptake and the incorporation of TGs into lipid droplets

(Lehrke *et al.*, 2005). LXRα regulates cholesterol homeostasis by responding to high cholesterol levels and by increasing fatty acid synthesis to increase the formation of cholesterol esters, rendering the excess cholesterol pool biologically inactive (Giguere, 1999; Pascussi *et al.*, 2008; Urquhart *et al.*, 2007). The effects of LXRα on fatty acid synthesis are mediated through the increased expression of SREBP-1c, a stimulator of lipogenesis. SREBP-1c is a transcription factor which has been previously associated with PI induced lipodystrophy (Bastard *et al.*, 2002; Telenti *et al.*, 2002). Chapters 2 and 3 of this thesis have already discussed the impact of LXRα gene expression on SREBP-1c so it will not be discussed further in this chapter.

This chapter has focused on key hepatic transporters involved in cholesterol homeostasis, bile acid metabolism and in glucose transport, although some of these transporters also play key metabolic roles in the periphery and the intestine. This chapter also hypothesises the relative impact that LXRα regulation has on cholesterol and bile acid transport with the intention to further probe its contribution in the deleterious metabolic complications associated with PI based HAART regimens.

ABCA1 is a mediator of the efflux of cholesterol and phospholipids to lipid poor apolipoproteins for the formation of nascent HDL-cholesterol (HDL-c) (Borst *et al.*, 2002). ABCA1 is a direct target of LXRα (Edwards *et al.*, 2002) the up-regulation of its gene expression by RTV was in line with the predicted outcome. Furthermore, it had been anticipated that this up-regulation could be blocked by antagonism of LXRα, as was observed in the RTV plus 22-(S)-hydroxycholesterol assays, supported by similar results in the biological knockdown (siRNA LXRα) assays.

Interestingly, the significant down-regulation of ABCA1 gene expression in the RTV plus 22-(S)-hydroxycholesterol assays provided an insight into other mechanisms regulating the gene and showed LXRα to be a primary regulator of ABCA1 gene expression. It is hypothesised that the co-repressor molecules NCoR and SMRT may be implicated in the down-regulation of ABCA1 expression in the presence of the LXRα antagonist, a regulatory role for these co-repressors has already been reported (Kalaany *et al.*, 2006). ABCA1 gene expression was not significantly altered in the presence of 0.1μM ATV, but at higher ATV concentrations significant down-regulation of ABCA1 which may be due to the involvement of SREBP-1c gene expression levels as it was established in Section 2.5.6 of this thesis that SREBP-1c expression is altered by both ATV and RTV incubations. Both FXR and LXRα regulate SREBP-1c and it had previously been implicated in the metabolic side effects due to PI use (Bastard *et al.*, 2002; Hui, 2003; Liang *et al.*, 2001; Osborne, 2000; Telenti *et al.*, 2002).

ABCB1 has previously been shown to be involved in the transport of both RTV and ATV, (Perloff *et al.*, 2005; Solon *et al.*, 2002). The down-regulation of ABCB1 gene expression by RTV at all concentrations and the increased gene expression in the presence of LXRα antagonism and knockdown suggests that LXRα may have been involved in ABCB1 gene regulation. It was hypothesised that LXRα may compete with other NRs, including may compete with other nuclear receptors, which may include PXR and HNF4α (Pascussi *et al.*, 2008; Tirona *et al.*, 2005), at the ABCB1 binding site. This would explain, in part, the differential gene expression response in the presence of known PXR agonists (including RTV and ATV), and LXRα agonists

(including RTV, as shown in these experiments) and the LXRα antagonist (22-(S)-hydroxycholesterol), although further investigations which were outside the scope of this current research would be required to probe this hypothesis. The current results suggested that there may be some merit in investigating the effects of PXR knockdown on the transporter response and to look at the effects of dual receptor knockdown for both PXR and LXRα on the gene expression of ABCB1 in the presence of RTV. Due to the time available for completion of this thesis this interesting further investigation is outside the scope of this thesis. The results from the current research suggest an inhibitory role for LXRα in respect of ABCB1 gene expression. ABCB1 was down-regulated by ATV at all concentrations, albeit to a lesser extent than the down-regulation observed in the RTV assays. As ATV has a more favourable lipid profile than RTV it did not warrant further investigation at this stage (Barreiro *et al.*, 2005; Feldt *et al.*, 2005; Flammer *et al.*, 2009).

The variable significant up-regulation and down-regulation of ABCB2, a key player in bile acid transporter, by RTV suggested that more than one mechanism was involved with its regulation. At 0.1μM RTV ABCB2 was significantly up-regulated but at higher RTV concentrations, ABCB2 gene expression was down-regulated. At lower RTV concentrations, the up-regulation of ABCB2 could be blocked by the use of the LXRα antagonist and by biological knockdown of LXRα, which implicated a role for ABCB2 regulation by LXRα. At higher RTV concentrations biological knockdown and chemical antagonism of LXRα resulted in only small differences in the regulation of ABCB2 suggesting that although LXRα may play a role in the regulation of this transporter it is not the master regulator of this gene.

ABCB11, the bile salt efflux pump, is regulated by FXR (Edwards *et al.*, 2002; Kalaany *et al.*, 2006). Significant up-regulation of ABCB11 gene expression by RTV was observed at 0.1μM (2.9 fold increase, P=<0.0001), which may be consistent with the observed significant up-regulation of FXR by RTV and its previously reported role in ABCB11 regulation (Edwards *et al.*, 2002; Kalaany *et al.*, 2006). Further probing of the role of FXR in this regulation would be required to confirm this hypothesis but this was outside the scope of this research. At higher concentrations of RTV, however, highly significant down-regulation of ABCB11 gene expression was evident at 1.0μM (1.9 fold decrease, P=<0.0001) and 10μM RTV (2.9 fold decrease, P=<0.0001). Furthermore the LXRα antagonist, 20μM 22-(S)-hydroxycholesterol, reversed the effects of RTV on ABCB11 suggesting that LXRα may also play a key role in the regulation of this gene. This hypothesis is further supported by the results of the siRNA LXRα knockdown assays which also diminished the effects of RTV on ABCB11.

Highly significant down-regulation of ABCC1 gene expression by RTV was observed at all RTV concentrations. In the presence of 20μM 22-(S)-hydroxycholesterol and the siRNA knockdown of LXRα, this down-regulation of ABCC1 gene expression by RTV was abolished at 0.1μM RTV plus 20μM 22-(S)-hydroxycholesterol, and diminished at higher RTV concentrations and in the siRNA samples in a concentration dependent manner. ABCC1 gene expression was also significantly down-regulated in the presence of all concentrations of ATV, suggesting a complex array of mechanisms involved in ABCC1 regulation.

Significant up-regulation of ABCC2 gene expression by RTV was observed at 0.1μM and 1.0μM, however, significant down-regulation was observed at 10μM RTV. These effects were diminished in the assays using the LXRα antagonist, 22-(S)-hydroxycholesterol, and in the biological knockdown assays. This suggests that LXRα may be a key regulator in the xenobiotic response of this gene, but that it was not the primary regulator of this gene which involves other NRs including PXR and FXR (Ho *et al.*, 2005; Wang *et al.*, 2008).

ABCG1, a key player in cholesterol transport, was significantly up-regulated by all concentrations of RTV. This up-regulation was diminished in the presence of the LXRα antagonist 22-(S)-hydroxycholesterol, and in the biological knockdown assays suggesting a role for LXRα in the regulation of ABCG1 gene expression. Furthermore another key player in cholesterol transport, ABCG4, was also significantly up-regulated by all concentrations of RTV, effects which could be diminished in the presence of 22-(S)-hydroxycholesterol and by biological knockdown of LXRα. This regulation by LXRα could provide an insight into some of the metabolic complications associated with HADL as genes on the pathways for both bile acid transport and cholesterol transport were up-regulated simultaneously is these assays.

Significant up-regulation of ABCG5 and ABCG8 gene expression was also observed in the RTV assays at all RTV concentrations. The effects of which could be abolished or diminished in the presence of 22-(S)-hydroxycholesterol or siRNA knockdown of LXRα. Both of these transporters have previously been cited as under the regulatory control of LXRα (Francis *et al.*, 2003). These experiments confirmed

that the gene expression of these metabolic transporters differs in response to RTV and ATV and that the actions of RTV can be blocked through modulation of LXRα gene expression. The results also provide further insight into the complex mechanisms by which LXRα regulates its targets in response to a RTV stimulus. This may provide valuable clues in the design of future ARVs and other drugs, as agonism of LXRα may result in the disruption of a cascade of metabolic genes and downstream targets. In Chapter 3, the observed effects of biological knockdown by siRNA of LXRα resulted in a down-regulation of LXRα gene expression in the presence of all RTV concentrations. This suggested a potential future therapeutic mechanism for antagonism of LXRα *in vivo,* if the issues which have previously been associated with increased production of liver triglycerides by LXRα agonists can be eliminated. This is discussed in more detail in Chapter 7, General Discussion. The results from the transporter assays also support this hypothesis.

Up-regulation of GLUT4 gene expression was observed at all concentrations of RTV. In the presence of 20μM 22-(S)-hydroxycholesterol, this was diminished at 0.1μM and 10μM RTV, and abolished at 1.0μM. The biological knockdown of LXRα confirmed these results implying a role for LXRα in the regulation GLUT4 gene expression. Although caution should be exercised when interpreting the GLUT 4 results as the overall basal and experimental expression levels of the gene were low in the Huh7 cells.

In summary, this chapter has described the specific impact of the PIs RTV and ATV on the gene expression of transporters involved in cholesterol homeostasis and bile acid metabolism, with specific focus on the role of LXRα in this regulation. It has

also discussed the impact of LXRα on the gene expression of the glucose transporter GLUT4; and its influence on the regulation of gene expression of this transporter in the presence of RTV. This chapter has hypothesised the role of multiple NRs, including LXRα, FXR, HNF4α and PXR, controlling regulation of key transporters, including ABCB1, and the need to consider whether future drugs act as LXRα agonists due to the disruptive effects it has on a cascade of metabolic genes and downstream targets. Inhibition of LXRα does show that some of these downstream effects can be modified.

CHAPTER 5

THE INFLUENCE OF A SINGLE NUCLEOTIDE POLYMORPHISM IN THE LIVER OXYSTEROL RECEPTOR-α (LXRα) GENE IN A HIV COHORT

5.1 Introduction

Dyslipidaemia and its related complications, including the increased risk of coronary artery disease, are growing public health concerns in both the HIV+ and HIV negative (HIV-) populations and an association with a genetic predisposition has been established (Alaynick, 2008; Dahlman *et al.*, 2006; Maes *et al.*, 1997; Pushpakom *et al.*). Long-term treatment with HAART is widely implicated with direct toxicity, which when associated with the complex interactions with HIV infection and combined with other factors, (including genetic predisposition, environmental) influence the risk of developing HADL. Morbidity and mortality outcomes for HIV+ individuals have greatly improved with successful treatment regimens but the onset of HADL significantly increases the risk of developing cardiovascular disease and associated metabolic complications (Kim *et al.*, 2006b; Mallon, 2007).

NRTIs are strongly associated with lipoatrophy and fat wasting, which is exacerbated by dual NRTI therapy (Mallal *et al.*, 2000; Mallon *et al.*, 2003). Insulin resistance is commonly associated with PIs and NRTIs (Falutz, 2007; Giguere, 1999; Lagathu *et al.*, 2005). PIs have been shown to impact several molecular pathways key to lipid metabolism including intra-nuclear transcription factors and the nuclear proteasome (Lagathu *et al.*, 2005; Mallon, 2007). There is a need for the development of newer ARVs, which overcome the problem of viral resistance, with improved safety profiles for long-term use in an aging HIV+ population, and reduced risk of HADL onset. Research into metabolic disorders, including HADL, has included

investigations into the role of NRs including LXRα (Cha *et al.*, 2007; Edwards *et al.*, 2002; Fiorucci *et al.*, 2007; Francis *et al.*, 2003; Tobin *et al.*, 2006).

In Chapters 2 and 3 of this thesis the impact of the PIs RTV and ATV on NR gene expression and the role played by LXRα in this was investigated and discussed. Chapter 2 discussed the implications of inhibition of LXRα gene expression by a chemical antagonist had on downstream target genes together with the hypothesis that LXRα functions as a master regulator of this downstream gene expression. Chapter 3 validated the role of LXRα by probing the influence of LXRα knockdown by siRNA, in the presence of RTV, on its own gene expression and that of its target genes to confirm the hypothesis. In these chapters the role of LXRα as an inducer of the gene expression of SREBP-1c, ApoA-I, ApoC-II and ApoC-III, although the latter may have been through an indirect mechanism via PPARα, was confirmed. LXRα was also confirmed as a down-regulator of PPARα and VLDLR gene expression.

5.1.1 Liver Oxysterol Activated Receptor-α (LXRα)

LXRα is expressed in the liver ([highest]), intestine, kidney, adipose tissue, macrophages, ovary, brain, lung and spleen. LXRα plays a critical role in cholesterol and bile acid homeostasis (Cha *et al.*, 2007; Hu *et al.*, 2003; Kalaany *et al.*, 2006). LXRα is involved in the up-regulation of CYP7A1 and is implicated in the increased expression of ABCA1, ABCG1, ABCG5 and ABCG8 (Eloranta *et al.*, 2005; Pascussi *et al.*, 2008; Tirona *et al.*, 2005). There is evidence that the effects of LXRα on fatty acid synthesis are mediated through the increased expression of SREBP-1c (Legry *et al.*, 2008), a transcription factor which has been previously associated with

PI induced lipodystrophy (Bastard *et al.*, 2002; Telenti *et al.*, 2002). LXRα agonists have been implicated in glucose homeostasis and metabolism (Cao *et al.*, 2003; Dahlman *et al.*, 2009; Grefhorst *et al.*, 2005). LXRα also represses PPARα and facilitates efflux of cholesterol from the periphery, including macrophages, through the increased expression of ABC transporters (Kalaany *et al.*, 2006; Pascussi *et al.*, 2008). PPARα induction leads to the repression of ApoC-III (Duval *et al.*, 2007; Feldman *et al.*, 2008; Yamada *et al.*, 2007). As LXRα plays a key role in numerous metabolic pathways it makes it an ideal candidate gene for the investigation of susceptibility to the metabolic complications associated with HADL.

5.1.2 LXRα Single Nucleotide Polymorphisms (SNPs) Associated with Metabolic Complications

Candidate SNPs were identified by a review of published LXRα SNP associations with dyslipidaemia in the general population in PubMed. The results were further refined to include only those with a minor allele frequency (MAF) ≥0.1 in Caucasian populations.

SNPs in LXRα have been implicated in a number of metabolic disorders including metabolic syndrome, dyslipidaemia and Type II diabetes mellitus (Dahlman *et al.*, 2009; Dahlman *et al.*, 2006; Legry *et al.*, 2008; Steffensen *et al.*, 2004). A specific association has been made with the heterozygous genotype of the rs2279238C/T SNP in LXRα with increased body mass index (BMI) and a hypothesised association in the homozygous T-allele population (rare TT genotype in cohort) with obesity (Dahlman *et al.*, 2006). A positive association has been made with the T allele and increased risk of a fatal cardiovascular event in a non-black population (Price *et al.*,

2011). The rs2279238C/T SNP in LXRα was considered to be a suitable candidate for investigation in a HIV+ cohort with associated HADL complications.

5.2 Aim

The aim of this chapter was to investigate the impact of a specific SNP in the LXRα gene which had previously been associated with metabolic complications (rs2279238CT, also known as C297T) in non-HIV+ individuals, in a HIV+ cohort which was treated with a HAART regimen which comprised PIs and NRTIs previously associated with HADL complications. The investigation focused on whether the SNP influenced the development of HADL in the HIV+ cohort.

5.3 Materials and Methods

5.3.1 Materials

Qiagen Miniprep Blood Kit, Qiagen Protease and Qiagen buffer AL, and Qiagen spin column were purchased from Qiagen Limited, (Crawley, West Sussex, UK). Pico Green dsDNA Quantitation Reagent was purchased from Invitrogen Limited (Paisley, UK). Taqman universal PCR master mix and inventoried Taqman allelic discrimination genotyping assay for LXRα rs2279238C/T were purchased from Applied Biosystems, (Warrington, UK). The 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, California, USA). Molecular grade Ethanol, Molecular Grade Isopropanol, RNAse free water, DNAse free water, and 2-mercaptoethanol were purchased from Sigma Aldrich Co. Limited (Irvine, Ayrshire, UK).

5.3.2 Patients

A United Kingdom (UK) cohort of HIV+ patients in this study were recruited from two centres in the UK (North Manchester General Hospital and the MRC Clinical Trials Unit). All subjects provided consent for blood donation/DNA samples and subsequent genetic analysis. The studies were approved by the local ethics committees. Patients were recruited as they consecutively attended the clinics during the period between 1998 and 2004. The diagnosis of HADL was based on the clinician's confirmation of patient self-report or on the clinician's observation alone after start of antiretroviral therapy, which had a PI backbone. HADL was defined as either lipoatrophy (fat wasting of arms, face, legs, or buttocks) or fat accumulation in the abdomen, or over the dorsocervical region or breasts, and presence of associated metabolic abnormalities. DNA samples were obtained from 172 HAART-treated HIV+ patients: 131 with HADL (HADL+) and 41 without HADL (HADL−). The inclusion criteria for the study were: age >18 years; Caucasian ethnicity and PI intake (including RTV) for at least 12 months before recruitment. HIV+ patients who met the above criteria but did not develop any features of HADL after 12 months of protease inhibitor treatment were recruited as controls. All the patients recruited were evaluated by the clinician at least three times over the course of a year. The following data were collected: age, sex, HADL status, concomitant ARVs, and baseline lipid parameters.

DNA samples were also obtained from 170 Caucasian HIV- healthy individuals with no known infectious or immune diseases to establish the population frequency of the

allelic variants investigated. Demographic data for the healthy controls was not retained. Study cohort demographics are as shown in Table 5.1 below.

Table 5.1: UK Patient Cohort Demographics for HADL LXRα Polymorphism Study

Patient Status	Patient Numbers	Age (Years) Median (Range)	Gender %
HADL+	131	40 (29-68)	80 (M), 20 (F)
HADL-	41	41 (24-65)	85.7 (M), 14.3 (F)
Healthy Controls	170	Not known	Not Known

The PIs administered included IDV, RTV, SQV, nelfinavir, amprenavir and lopinavir. All patients included in this study had also received NRTIs which included stavudine, zidovudine and lamivudine.

5.3.3 Extraction of Genomic DNA from Whole Blood

Total genomic DNA was extracted from whole blood using the Qiagen Miniprep Blood Kit. Whole blood (200µl) was mixed with Qiagen Protease (20µl) and buffer AL (200µl) and pulse vortexed (15s). Samples were incubated (10 min, 56°C) and pulse vortexed (15s) before the addition of ethanol (200µl) and transferred to a Qiagen spin column. The spin column was centrifuged (6000 x g, 1 min, 4°C) and the flow-through discarded. Buffer AW1 (500µl) was added and the column centrifuged (6000 x g, 1 min, 4°C) discarding the flow-through. A further wash with buffer AW1 was carried out (500µl, 17000 x g, 1 min, 4°C). Buffer AE (200µl) was then added and the spin column was incubated (5 min, 22°C) before centrifugation (17000 x g, 1 min, 4°C) and collection of flow-through in clean collection tubes. Samples were diluted (1:100), analysed and quantified using the PicoGreen® dsDNA

Quantitation Reagent, validated by standard curve and were normalised to a concentration of 20ng/μl by the addition of DNAse free water.

5.3.4 LXRα Genotyping by Taqman Allelic Discrimination Assay

DNA isolated from whole blood was genotyped for the rs2279238CT (SNP within LXRα by Taqman allelic discrimination assay. The assay involves qPCR of both C and T alleles by the same primers in the presence of two probes, one specific for each allele, and labelled with different reporter dyes (FAM and VIC) between which the detector can distinguish. Following amplification, the 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, California, USA) reports on which wavelength fluorescence is detected. If amplification is detected (i.e. the cycle threshold is exceeded) in only one wavelength, the sample is considered homozygous for whichever allele the probe fluorescing at that wavelength is specific. Samples in which amplification is detected in both wavelengths are considered heterozygous. The reaction mixture consisted of Taqman universal PCR master mix (2.5μl), LXRα rs2279238CT primer and probe mix (0.25μl), nuclease-free water (0.25μl) and DNA normalised stock (2μl) per well of a 384 well optical reaction plate. Thermal cycling conditions consisted of 1 cycle at 95°C for 10 minutes, then 40 cycles of 92°C for 15 seconds and a combined annealing/extending temperature of 60°C for 1 minute.

5.4. Statistical Analysis

All data are given as frequency number (percentage), unless otherwise stated. Genotypes were tested for Hardy–Weinberg equilibrium by χ^2 (Chi-square) test of observed versus predicted (from allele frequency) genotype frequencies. Statistical significance was assessed using the χ^2 test and Fishers exact tests for subset analysis.

Spearman's rank correlations were used to assess the correlation of age and gender with HADL status, baseline total cholesterol, baseline glucose and baseline triglyceride levels. Univariate and multivariate associations were assessed with SPSS v17 (SPSS Inc., Chicago, IL, USA) using backward logistic regression. A statistical trend was defined as a P value <0.1. Statistical significance was set at P<0.05.

5.5. Results

5.5.1 Quantification of Genomic DNA

Total genomic DNA samples were diluted (1:100), analysed and quantified using the PicoGreen® dsDNA Quantitation Reagent, validated by standard curve prepared from known DNA concentrations at 1.5625ng/µl, 3.125ng/µl, 6.25ng/µl, 12.5ng/µl, 25ng/µl and 50ng/µl as shown in Figure 5.1 below.

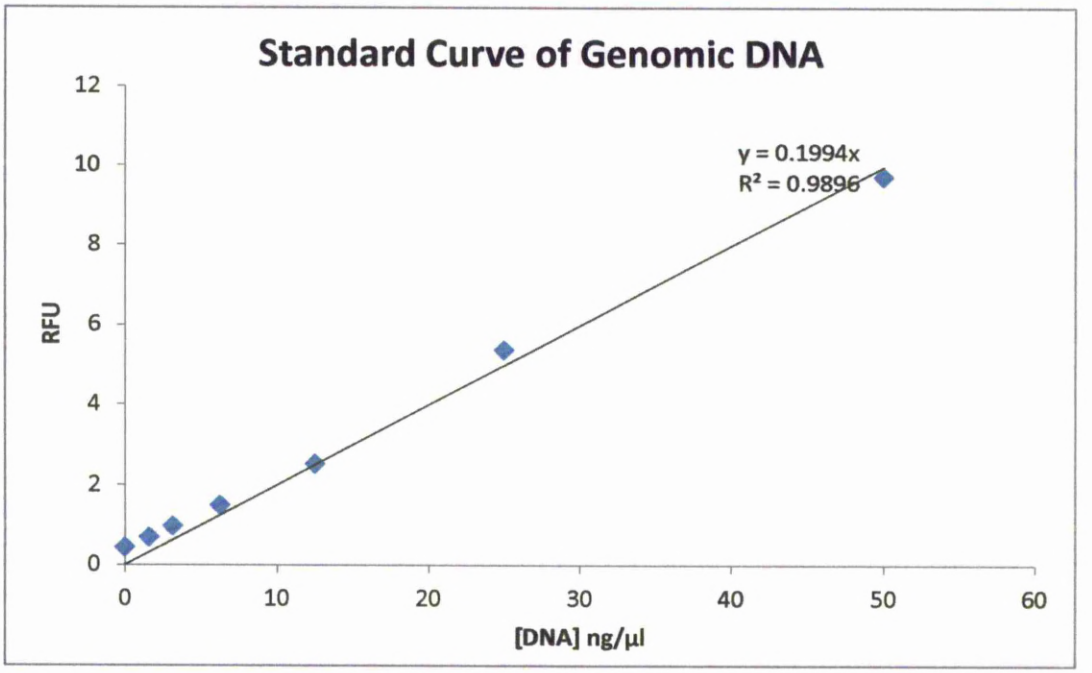


Figure 5.1: Standard Curve of known concentration genomic DNA used for quantification of sample genomic DNA.

5.5.2 LXRα Genotyping by Taqman Allelic Discrimination Assay

Patient demographics and characteristics were outlined in Section 5.3.2 and Table 5.1. The distribution of the genotypes for the LXRα rs2279238C/T SNP included 239 individuals with the CC genotype (95 HADL+, 35 HADL- and 109 healthy volunteers), 94 heterozygotes with the CT genotype (32 HADL+, 6 HADL- and 56 healthy volunteers) and 9 homozygous TT individuals (4 HADL+, no HADL- and 5 healthy volunteers). Allele frequencies for the LXRα rs2279238C/T SNP were confirmed to be in Hardy-Weinberg equilibrium and are summarised in Table 5.2 below.

Table 5.2: Summary allelic frequencies and Hardy-Weinberg analysis for rs2279238CT SNP in LXRα.

Hardy-Weinberg equilibrium for Manchester/MRC Lipodystrophy study

The 5% significance level for 2 degrees of freedom for $\chi 2$ value is 3.84 or P value < 0.05

(n=131/41/170)

SNP	Genotype frequencies (n=131/41/170)			Genotype frequencies (%)			Allele frequencies (n=131/41/170)		Allele frequencies %		χ2 test	P value
									p	q		
LXRα C297T HADL+	CC	CT	TT	CC	CT	TT	C	T				
	95	32	4	72.5	24.4	3.1	222	40	84.7	15.3	0.1870	0.9107
LXRα C297T HADL-	CC	CT	TT	CC	CT	TT	C	T				
	35	6	0	85.4	14.6	0.0	76	6	92.7	7.3	0.2374	0.8881
LXRα C297T Healthy Volunteers	CC	CT	TT	CC	CT	TT	C	T				
	109	56	5	64.1	32.9	2.9	274	66	80.6	19.4	0.2547	0.8804

Figure 5.2 below shows the cycle threshold analysis of the allelic discrimination assay. Figures 5.3, 5.4 and 5.5 are representative plots of the homozygous wild type CC, heterozygote CT and homozygous mutant TT respectively.

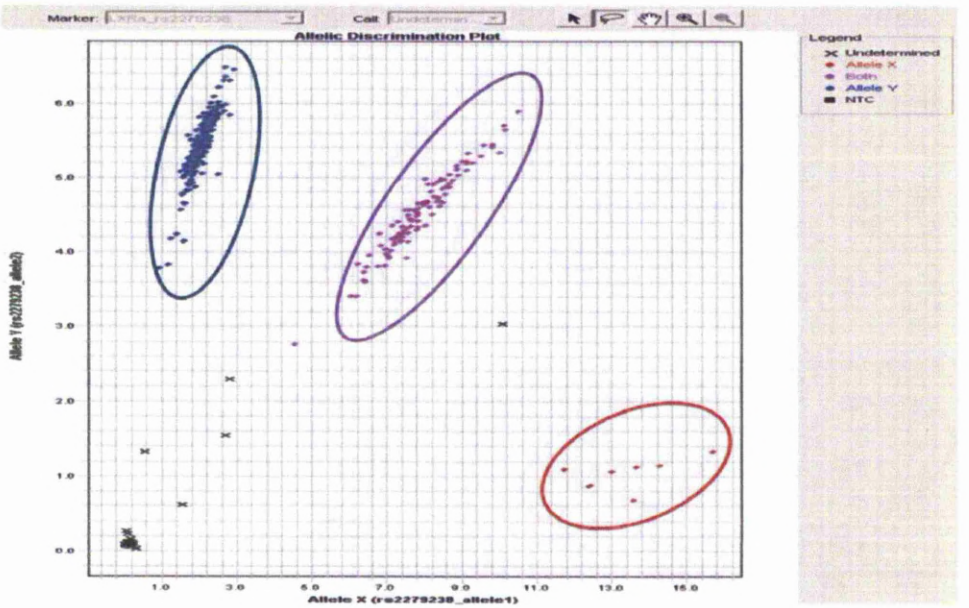


Figure 5.2: LXRα SNP rs2279238C/T: Cycle Threshold analysis of allelic discrimination assay by qPCR

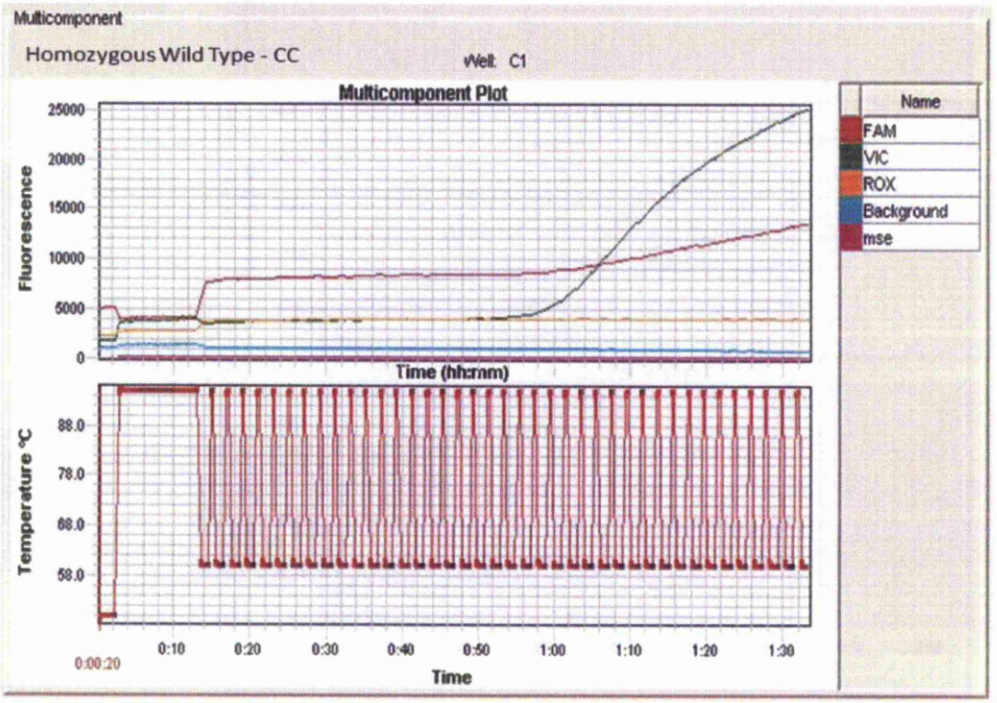


Figure 5.3: LXRα SNP rs2279238C/T: Representative Plot of Homozygous Wild Type-CC by allelic discrimination assay by qPCR, n=1

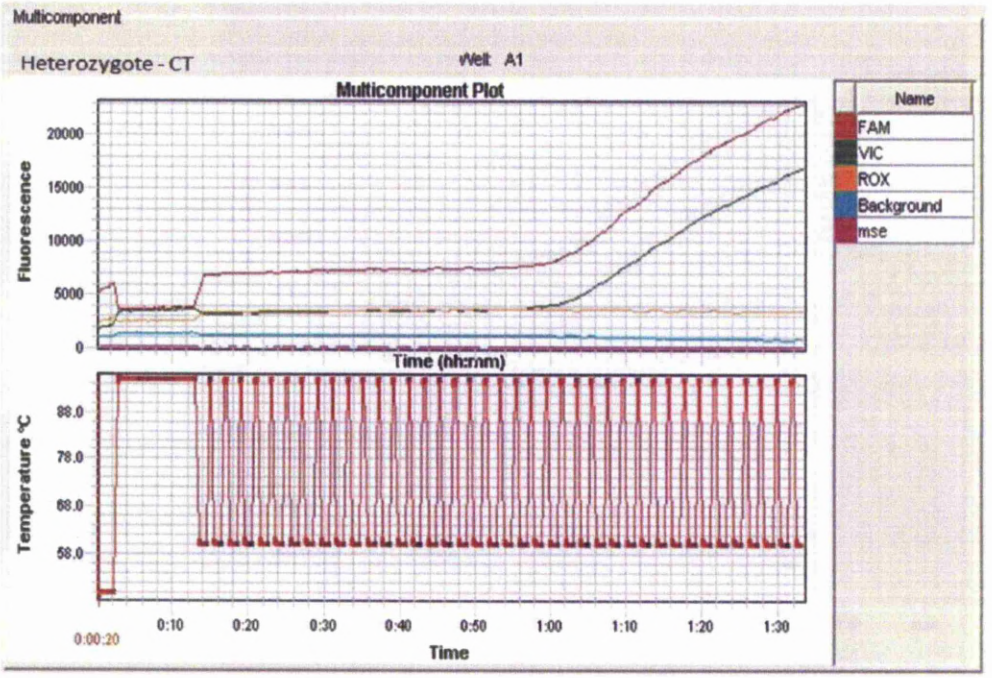


Figure 5.4: LXRα SNP rs2279238C/T: Representative Plot of Heterozygous-CT by allelic discrimination assay by qPCR, n=1

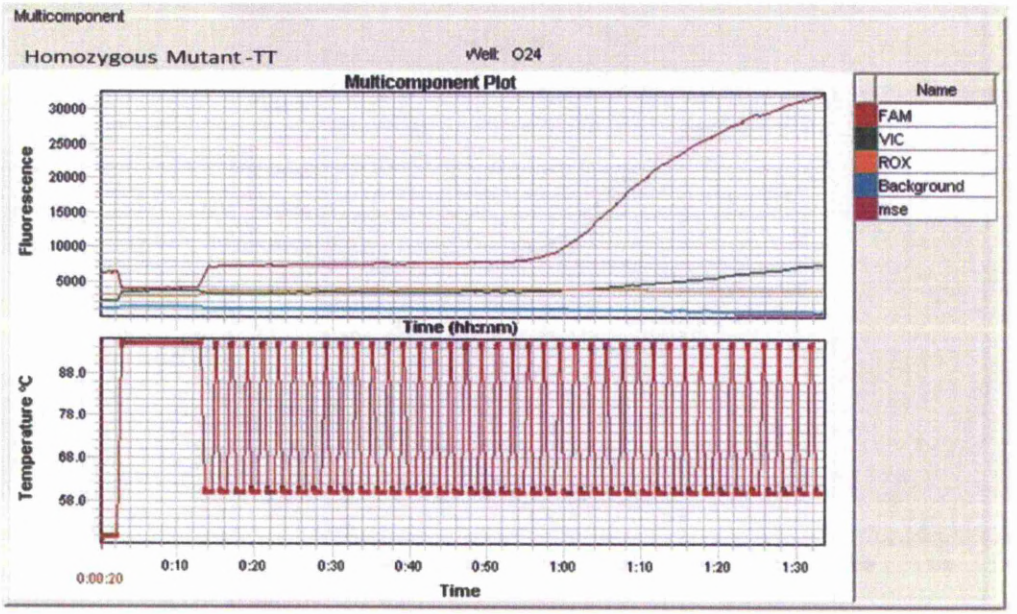


Figure 5.5: LXRα SNP rs2279238C/T: Representative Plot of Homozygous Mutant-TT by allelic discrimination assay by qPCR, n=1

There were no significant associations with any of the genotypes with baseline lipid parameters (Table 5.3 below). A trend was observed with HADL+ status and baseline triglyceride levels >200mg/dl (P=0.069), and with baseline glucose levels >100mg/dl (P=0.087). A significant association was observed with high baseline cholesterol levels ≥240mg/dl and HADL+ status (P=0.001) and age (P=0.009). Summary univariate and multivariate outcomes are detailed below, Table 5.3. Spearman's rank correlations did not confirm a correlation of age and/or gender with HADL status, baseline total cholesterol, baseline glucose and baseline triglyceride levels. A moderate correlation between HADL status and baseline total cholesterol levels (r=0.319) was confirmed by Spearman's rank correlation. Multivariate logistic regression did not confirm significant associations with any of the genotypes in the either the HADL+ or HADL- cohorts. Subset analysis of HADL+ and HADL-

cohorts underpinned this finding, however, a trend was observed when the alleles were compared in the same groups (P=0.09). A significant association was observed when HADL- and healthy volunteer cohorts were analysed (P=0.0012) whilst the power of this association increased when the alleles in the same cohorts were analysed (P=0.008). Frequency distribution analysis confirmed that there were differences between the phenotype outcomes for the three genotypes in that the TT genotype was not observed in the HADL- cohort. Individuals with at least one copy of the rare T allele were more prevalent in the HADL+ and healthy volunteer cohorts, see Figure 5.6 below.

Table 5.3: Summary univariate and multivariate regression analysis of rs2279238CT SNP in LXRα together with Spearman's Rank correlation analysis with HADL status. Significance = P=<0.05, Trend = P=<0.1.

	Baseline Cholesterol ≥240mg/dl	**Baseline Triglycerides ≥200mg/dl**	**Baseline Glucose ≥100mg/dl**	**Age**	**Gender**	**HADL+**
Univariate P Value	0.05	0.069	0.087	0.633	0.312	0.648
Multivariate P Value	0.033	0.082	0.067	0.009	0.238	0.001
95% Confidence Interval	0.208-0.802	0.856-1.595	0.000-0.368	0.005-0.037	-0.537-0.235	0.193-0.786
Spearman's Rho (r=)	0.319	0.091	0.073	-0.010	0.059	

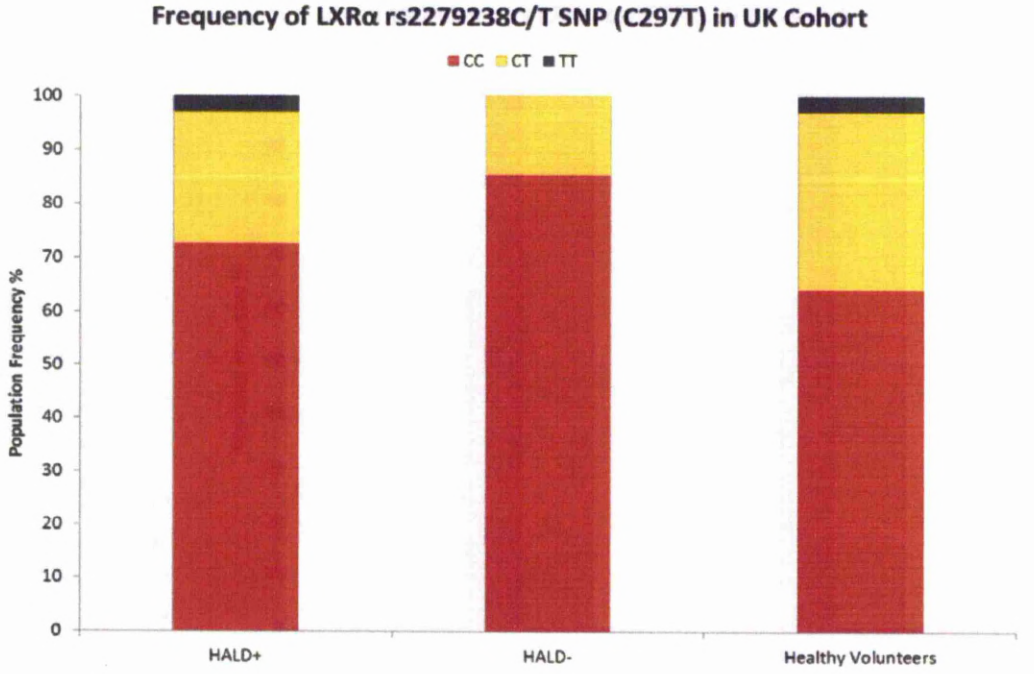


Figure 5.6: LXRα SNP rs2279238C/T: Frequency distribution (%) of genotypes for the C297T SNP in HIV+ Patient Cohort and Healthy Volunteer populations, P = 0.645. HADL+ = HIV+ with HADL symptoms, HADL- = HIV+ without HADL symptoms.

5.6 Discussion

This chapter sought to validate the importance of a known polymorphism in the LXRα gene which had previously been associated with lipid abnormalities and cardiovascular risk in non-HIV+ populations (Dahlman *et al.*, 2009; Legry *et al.*, 2008; Price *et al.*, 2011) in a HIV+ cohort with confirmed HADL complications. The investigation determined that there were no significant associations with any of the CC, CT and TT genotypes of the rs2279238C/T SNP in either the HADL+ or HADL- cohorts, although it should be noted that the rare TT genotype was completely absent in the HADL- cohort. Subset analysis of the allele frequency in the HADL+ and HADL- cohorts revealed a trend associated with the T allele and HADL symptoms (P=0.09). When the HADL- cohort was compared to the healthy

volunteer cohort a significant association was observed which suggested a protective role for the CC genotype in this cohort (P=0.0012) whilst the power of this association increased when the alleles in the same cohorts were analysed (P=0.008) with an odds ratio of 3. This suggested that the polymorphism was 3 times more likely in healthy volunteers than in the HADL- cohort which supported the hypothesis that absence of the polymorphism may be protective against HADL symptoms. It should also be noted that the mutant TT genotype was rare in the patient cohort. Baseline lipid parameters and patient demographics were not obtained for the healthy volunteers and some HADL patients had incomplete lipid profiles. The parameters used for analysis of the lipid levels in the HADL cohort were in line with the recommendations of the National Institute of Health (NIH), USA (Public-Health, 2001). Further investigations in a larger population would need to be undertaken to establish whether the association with the TT genotype previously observed in HIV absent populations (Dahlman *et al.*, 2009; Legry *et al.*, 2008; Price *et al.*, 2011) influences HADL outcomes in non-Caucasian populations or is gender specific in a HIV+ cohort, as this patient group was wholly Caucasian and predominantly male.

Weak associations between baseline lipid parameters; total cholesterol ≥240mg/dl (P=0.05); triglycerides ≥200mg/dl (P=0.069); and blood glucose ≥100mg/dl (P=0.087) were observed in the HADL+ cohort, although these associations were not linked to a specific genotype. Power for these associations increased in the multivariate analysis when increasing age of the patients was correlated with HADL status and the baseline parameters, however, these did not correlate with a specific

genotype. Total cholesterol ≥240mg/dl was established in 39/95 HADL+ homozygous wild type (CC) patients, 15/32 heterozygotes (CT) and 1/4 homozygous rare allele (TT) patients. Triglycerides ≥200mg/dl were determined in 42/95 (although TG data was missing for 25/95 patients in this category) HADL+ homozygous wild type (CC) patients, 13/32 (although TG data was missing for 10/32 patients in this category) heterozygotes (CT) and 1/4 homozygous rare allele (TT) patients (TG data was missing for the other 3 patients in this category). Blood glucose ≥100mg/dl was detected in 20/95 (although glucose data was missing for 7/95 patients in this category) HADL+ homozygous wild type (CC) patients, 8/32 heterozygotes (CT) and 1/4 homozygous rare allele (TT) patients (glucose data was missing for the other 3 patients in this category). In order to validate these associations further investigations in a larger population would need to be undertaken to establish whether these parameters correlate with a specific genotype.

It is hypothesised that the LXRα SNP rs2279238C/T influences the risk of HADL development in Caucasian HIV+ populations, it may also be linked to increased risk of a fatal cardiovascular event where HADL complications are present. Survival outcome and follow-up data for this cohort were not recorded so the cardiovascular risk is unconfirmed. HADL is a multifactorial disease and in this chapter a single polymorphism in a single gene has been investigated. It is difficult to conclude that the presence of a single polymorphism in LXRα could predict future cardiovascular events in a HIV+ or HADL+ population, although it is possible to make associations with other SNPs in linkage disequilibrium with rs2279238C/T by use of the HapMap to establish the presence of multiple SNPs which contribute to the phenotype.

Research into the role of host genetics in the development of lipid abnormalities in response to ARVs continues with polymorphisms in ApoA-5, ApoC-III, ApoE and ABCA1 widely implicated (Owen *et al.*, 2008; Tarr *et al.*, 2007; Telenti *et al.*, 2008).

HIV is a chronic but manageable disease, as the HIV+ population ages the risk of developing metabolic disorders including diabetes mellitus and cardiovascular disease increases. In order to offset this risk, health care professionals need to consider metabolic complications, especially cardiovascular risk assessment, as a routine component of treatment of HIV+ individuals (Moyle, 2007). Knowledge of specific polymorphisms which increase this risk will provide an insight into future treatment regimens so that those individuals with the greatest risk of metabolic complications receive treatment which incorporates lipid sparing ARVs. This knowledge will also influence the future development of drugs to treat HIV as more is known about the complex mechanisms which underpin HADL and individual susceptibility to its development.

In summary, this chapter described the association of a known polymorphism in the metabolic gene LXRα which had previously been linked with lipid abnormalities in HIV- populations and the role that this polymorphism has had in influencing the development of HADL in a Caucasian HIV+ cohort together with the potential influence that these findings may have as future predictors of HADL risk.

CHAPTER 6

THE INFLUENCE OF SINGLE NUCLEOTIDE POLYMORPHISMS IN ENDOGENOUS CHOLESTEROL METABOLISM GENES IN AN ITALIAN HIV COHORT

6.1 Introduction

The risk of developing HIV/HAART associated dyslipidaemic lipodystrophy (HADL) has emerged as a major long term concern for HIV+ individuals treated with HAART. The onset of HADL greatly increases the risk of developing cardiovascular disease and associated metabolic complications (Kim *et al.*, 2006b; Mallon, 2007). Protease inhibitor (PIs) containing regimens in particular have been associated with an increased cardiovascular risk which is in part mediated by dyslipidaemia (Friis-Moller *et al.*, 2003; Friis-Moller *et al.*, 2008). The three major classes of antiretrovirals: PIs, nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) have all been implicated in HADL, although PIs are most widely implicated with symptoms occurring in 60-80% of HIV+ individuals on PI based regimens (Behrens *et al.*, 2000; Carper *et al.*, 2008; Carr, 2000; Carr *et al.*, 1998; Falutz, 2007; Mallon, 2006). Ritonavir (RTV) is no longer used as a PI in HAART but is used at low dose to boost the activity of other PIs through its inhibition of the cytochrome P450 (CYP) enzyme, CYP3A4. RTV has no intrinsic antiviral activity at low dose but it has been widely implicated in hypertriglyceridaemia (Kim *et al.*, 2006b; Mallon, 2007; Purnell *et al.*, 2000).

HADL may include increased low-density lipoprotein cholesterol (LDL-C) levels, decreased high-density lipoprotein cholesterol (HDL-C) levels, and hypertriglyceridaemia (Fontas *et al.*, 2004; Periard *et al.*, 1999; Young *et al.*, 2005). The severity and category of dyslipidaemia may vary with the HAART regimen used. For example, hypertriglyceridaemia is frequently associated with RTV

containing regimens (Fontas *et al.*, 2004; Periard *et al.*, 1999), whereas increased LDL-C levels are associated with multiple classes of antiretrovirals (ARVs) including PIs and NNRTIs, especially efavirenz (Fontas *et al.*, 2004; Periard *et al.*, 1999; van Leth *et al.*, 2004). Hypercholesterolaemia, hypertriglyceridaemia and elevated apolipoprotein B (ApoB) levels are more widely associated with the use of PI based regimens (Lekakis *et al.*, 2008; Liang *et al.*, 2001; Purnell *et al.*, 2000; Tsiodras *et al.*, 2000). RTV has also been associated with enhanced formation of very low density lipoproteins (VLDL), whilst saquinavir is associated with a decrease in lipoprotein lipase (LPL) activity and increased mobilisation of lipid stores (Purnell *et al.*, 2000; Ranganathan *et al.*, 2002; Reeds *et al.*, 2003).

HADL does not, however, develop in all HIV+ patients despite similar HAART exposure and comparable immunological and demographic characteristics. This may implicate host genetic factors playing a significant role in metabolic complications including HADL.

6.1.1 Single Nucleotide Polymorphisms (SNPs) Associated with Metabolic Complications

Candidate SNPs were identified by a review of published SNP associations with dyslipidaemia in the general population in PubMed, excluding those which had previously been extensively associated in the HIV+ population. The results were further refined to include only those with a minor allele frequency (MAF) ≥ 0.1 in Caucasian populations.

Two SNPs in the low density lipoprotein receptor (LDLR) were identified which had previously been associated with increased LDL-C and an increased risk of cardiovascular events, rs1529729C/T and rs2228671C/T (Aulchenko *et al.*, 2009; Kathiresan *et al.*, 2008; Linsel-Nitschke *et al.*, 2008). The C allele in both SNPs was associated with increased LDL-C (Kathiresan *et al.*, 2008; Linsel-Nitschke *et al.*, 2008), whilst the risk of cardiovascular events further increased when the rs1529729C/T SNP was present in association with other SNPs including the rs693A/G and rs7575840G/T SNPs found in the apolipoprotein-B (ApoB) gene, which were also associated with increased LDL-C levels (Kathiresan *et al.*, 2008). The C allele for the SNP rs2278986C/T in the scavenger receptor class B1 (SCARB1, also known as SRB1)) gene has been implicated in reduced SRB1 protein levels which are inversely associated with HDL-C levels, with the prevalence for higher HDL-C levels being greater in females with hyperalphalipoproteinaemia (West *et al.*, 2009).

The rs2230806G/A SNP in the ATP binding cassette protein A1 (ABCA1, formerly known as the cholesterol efflux regulatory protein, CERP) has been associated with elevated LDL-C levels in men with the AA genotype (Abellan *et al.*). Females with the AA genotype for the rs1893590A/C SNP in the ATP binding cassette protein G1 (ABCG1) have been shown to have decreased HDL-C levels (Abellan *et al.*). The T allele of the rs6544713C/T SNP in the ATP binding cassette protein G8 (ABCG8) has been associated with elevated LDL-C levels and an increased risk of carotid artery disease (Ronald *et al.*, 2009).

6.1.2 Metabolic Parameters

Total cholesterol is defined as low if it is <200mg/dl in whole blood. At 200-239mg/dl the patient is described as borderline hypercholesterolaemia and values ≥240mg/dl are determined to be high total cholesterol (Public-Health, 2001). The parameters for HDL-C differ in males and females within the following ranges: Low HDL-C in men is <40mg/dl whereas <50mg/dl is considered low in women. Average HDL-C levels are in the range 40-50mg/dl for males and 50-60mg/dl in females, with high HDL-C levels >50mg/dl in men and >60mg/dl in women, the latter category is regarded as beneficial (Public-Health, 2001).

Optimal LDL-C levels are <100mg/dl for both males and females. Levels in the range 100-129mg/dl are considered near optimal whilst those in the range 130-159mg/dl are considered to be borderline high. High LDL-C is in the range 160-189mg/dl whereas >190mg/dl is considered to be very high LDL-C (Grundy *et al.*, 2004; Public-Health, 2001).

Normal triglyceride (TG) levels are <150mg/dl, with borderline high levels in the range 150-199mg/dl. High TG levels are in the range 200-499mg/dl with very high TG levels >500mg/dl (Public-Health, 2001).

Blood glucose levels are considered normal when <100mg/dl, whereas 100-126mg/dl is considered to represent impaired fasting or pre-diabetes. Individuals with diabetes would have blood glucose levels >126mg/dl.

6.2 Aim

The aim of this chapter was to investigate the impact of specific polymorphisms in genes which had previously been associated with metabolic complications (ABCA1, LDLR, SCARB1, ABCG1, ABCG8 and ApoB) in non-HIV+ individuals, in a HIV+ cohort with known HADL symptoms who had been switched from a RTV-ATV boosted regimen to an un-boosted ATV regimen.

6.3 Materials and Methods

6.3.1 Materials

Qiagen Miniprep Blood Kit, Qiagen Protease, Qiagen buffer AL, and Qiagen spin columns were purchased from Qiagen Limited, (Crawley, West Sussex, UK). Taqman universal master mix, inventoried Taqman allelic discrimination genotyping assays for ABCA1 rs2230806G/A, LDLR rs1529729C/T and rs2228671C/T, SCARB1 rs2278986C/T, ABCG1 rs1893590A/C, ABCG8 rs6544713C/T, and ApoB rs693A/G and rs7575840G/T were purchased from Applied Biosystems, (Warrington, UK). Pico Green dsDNA Quantitation Reagent was purchased from Invitrogen Limited (Paisley, UK). Molecular grade Ethanol and DNAse free water were purchased from Sigma Aldrich Co. Limited (Irvine, Ayrshire, UK). The DNA Engine Opticon 2 and Chromo 4 systems were from MJ Research Inc. (USA).

6.3.2 Patients

An Italian cohort of Caucasian HIV+ patients from the Department of Infectious Diseases, University of Turin, Italy (n = 32, 17 male and 15 female) participated in this study. Inclusion criteria for the cohort was as follows: receipt of un-boosted atazanavir (400 mg once per day), age >18 years, and no receipt of drugs known to

alter plasma concentration of atazanavir (e.g. proton pump inhibitors or non-nucleoside reverse-transcriptase inhibitors). The following data were collected: age, sex, body weight, total cholesterol (mg/dl), HDL-cholesterol (mg/dl), blood glucose (mg/dl), LDL-cholesterol (mg/dl), triglycerides (mg/dl), (Table 6.1, below) concomitant antiretroviral drugs, and other medications. Parameters were measured at baseline (week 0, last date for RTV boosted ATV), with follow up values taken at weeks 4, 12, 24, 36, 48, 60, 72, 84 and 96. All subjects provided consent for blood donation and the study was approved by the local ethics committee.

Table 6.1: Italian Patient Cohort Demographics for Cholesterol Transport Genes SNP Study

Patient Numbers	32
Age (Years) Median (Range)	45 (34-64)
Height (cm) Median (Range)	168 (150-185)
Weight (kg) Median (Range)	60 (41-90)
Baseline Total Cholesterol (mg/dl) Median (Range)	137 (101-323)
Baseline HDL Cholesterol (mg/dl) Median (Range)	30 (24-62)
Baseline Blood Glucose (mg/dl) Median (Range)	95 (67-128)
Baseline LDL Cholesterol (mg/dl) Median (Range)	63 (51-216)
Baseline Triglycerides (mg/dl) Median (Range)	217 (74-629)
Gender %	53 (M), 47 (F)

6.3.3 Extraction of Genomic DNA from Whole Blood

Total genomic DNA was extracted from whole blood using the Qiagen Miniprep Blood Kit. Whole blood (200µl) was mixed with Qiagen Protease (20µl) and buffer AL (200µl) and pulse vortexed (15s). Samples were incubated (10 min, 56°C) and pulse vortexed (15s) before the addition of ethanol (200µl) and transferred to a

Qiagen spin column. The spin column was centrifuged (6000 x g, 1 min, 4°C) and the flow-through discarded. Buffer AW1 (500µl) was added and the column centrifuged (6000 x g, 1 min, 4°C) discarding the flow-through. A further wash with buffer AW1 was carried out (500µl, 17000 x g, 1 min, 4°C). Buffer AE (200µl) was then added and the spin column was incubated (5 min, 22°C) before centrifugation (17000 x g, 1 min, 4°C) and collection of flow-through in clean collection tubes. Samples were diluted (1:100), analysed and quantified using the PicoGreen® dsDNA Quantitation Reagent, validated by standard curve and were normalised to a concentration of 20ng/µl by the addition of DNAse free water.

6.3.4 Genotyping by Taqman Allelic Discrimination Assay

The following genes and associated SNPs were genotyped by Taqman Allelic Discrimination Assay: ABCA1 (rs2230806G/A SNP), LDLR (rs1529729C/T and rs2228671C/T SNPs), SCARB1 (rs2278986C/T SNP), ABCG1 (rs1893590A/C SNP), ACG8 (rs6544713C/T SNP), and ApoB (rs693A/G and rs7575840G/T SNPs) in DNA isolated from whole blood as described below.

The assay involves qPCR of both G (C) and A (T) alleles by the same primers in the presence of two probes, one specific for each allele, and labelled with different reporter dyes (FAM and VIC) between which the detector can distinguish. Following amplification, the Real Time PCR machine (Opticon 2/Chromo4) reports on which wavelength fluorescence is detected. If amplification is detected (i.e. the cycle threshold is exceeded) in only one wavelength, the sample is considered homozygous for whichever allele the probe fluorescing at that wavelength is specific. Samples in

which amplification is detected in both wavelengths are considered heterozygous. The reaction mixture consisted of Taqman universal master mix (12.5 μl), specific Taqman Allelic Discrimination Assay primer and probe mix (1.25 μl), water (9.25 μl) and DNA normalised stock (2 μl) per well of a 96 well optical reaction plate. Thermal cycling conditions consisted of 46 cycles with a combined annealing/extending temperature of 60°C.

6.4. Statistical Analysis

All data are given as frequency number (percentage), unless otherwise stated. Genotypes were tested for Hardy–Weinberg equilibrium by χ^2 test of observed versus predicted (from allele frequency) genotype frequencies. Statistical significance was assessed using the χ^2 test. Univariate and multivariate associations were assessed with SPSS v17 (SPSS Inc., Chicago, IL, USA) using backward logistic regression. A statistical trend was defined as a P value <0.1. Statistical significance was set at $P<0.05$.

6.5. Results

6.5.1 Quantification of Genomic DNA

Total genomic DNA samples were diluted (1:100), analysed and quantified using the PicoGreen® dsDNA Quantitation Reagent, validated by standard curve prepared from known DNA concentrations at 1.5625ng/μl, 3.125ng/μl, 6.25ng/μl, 12.5ng/μl, 25ng/μl and 50ng/μl as shown in Figure 6.1 below.

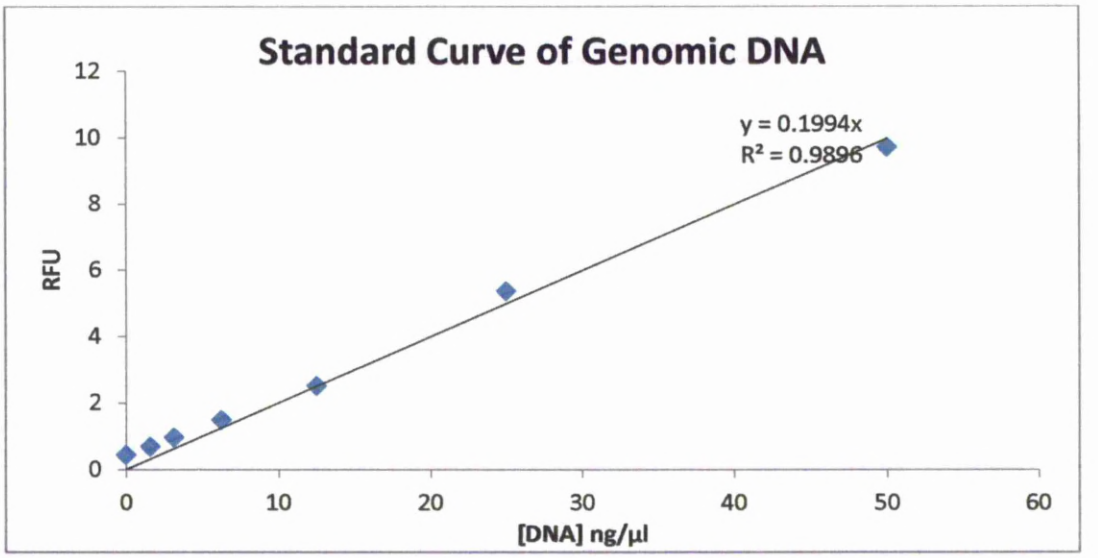


Figure 6.1: Standard Curve of known concentration genomic DNA used for quantification of sample genomic DNA.

6.5.2 Summary Allele Frequencies in HIV+ Cohort

Allele frequencies for ABCA1 rs2230806G/A, LDLR rs1529729C/T and rs2228671C/T, SCARB1 rs2278986C/T, ABCG1 rs1893590A/C, ACG8 rs6544713C/T, ApoB rs693A/G and rs7575840G/T were in Hardy-Weinberg equilibrium (Table 6.2).

Table 6.2: Summary allelic frequencies and Hardy-Weinberg analysis for SNPs in ABCA1, LDLR, SCARB1, ABCG1, ABCG8 and ApoB. The 5% significance level for 2 degrees of freedom for $\chi 2$ value is 3.84 or P value < 0.05

SNP	Genotype frequencies			Genotype frequencies(%)			Expected genotype frequencies			Allele frequencies		$\chi 2$ test	P value
	(n=32)						(n=32)			p	q		
ABCA1 G1051A - rs2230806GA	GG	GA	AA	GG	GA	AA	GG	GA	AA				
	11	17	4	34.4	53.1	12.5	11.88	15.23	4.88	0.6094	0.3906	0.218508	0.8965
LDLR (SMARCA4) rs1529729 C/T	CC	CT	TT	CC	CT	TT	CC	CT	TT				
	5	13	14	15.6	40.6	43.8	4.13	14.73	13.13	0.3594	0.6406	0.218518	0.8965
SCARB1-rs2278986 C/T	CC	CT	TT	CC	CT	TT	CC	CT	TT				
	2	22	8	6.3	68.8	25.0	5.28	15.44	11.28	0.4063	0.5938	3.187427	0.2032
LDLR-rs2228671 C/T	CC	CT	TT	CC	CT	TT	CC	CT	TT				
	30	2	0	93.8	6.3	0.0	30.03	1.94	0.03	0.9688	0.0313	0.032258	0.984
ABCG1-rs1893590 A/C	AA	AC	CC	AA	AC	CC	AA	AC	CC				
	18	13	1	56.3	40.6	3.1	18.76	11.48	1.76	0.7656	0.2344	0.317681	0.8531
ABCG8-rs6544713 C/T	CC	CT	TT	CC	CT	TT	CC	CT	TT				
	13	14	5	40.6	43.8	15.6	12.50	15.00	4.50	0.625	0.375	0.070602	0.9653
ApoB-rs693 A/G	AA	AG	GG	AA	AG	GG	AA	AG	GG				
	4	15	13	12.5	46.9	40.6	4.13	14.73	13.13	0.3594	0.6406	0.005217	0.9974
ApoB-rs7575840 G/T	GG	GT	TT	GG	GT	TT	GG	GT	TT				
	22	10	0	68.8	31.3	0.0	22.78	8.44	0.78	0.8438	0.1563	0.927295	0.629

6.5.3 ABCA1 Genotyping and Association with Metabolic Parameters

The distribution of the genotypes for the rs2230806G/A SNP within ABCA1 included 4 individuals with the AA genotype (1 male and 3 female), 17 heterozygotes with the GA genotype (8 male and 9 female) and 11 homozygous GG individuals (8 male and 3 female). Univariate analysis of the patient data did not confirm any significant associations between the patient genotype and the baseline or week 24 follow-up lipid parameters, Table 6.3.

Table 6.3: Univariate analysis of ABCA1 rs2230806G/A SNP in Italian Patient Cohort on Metabolic Parameters (n=32)

	GG	GA	AA	P Value
Patient Numbers	11	17	4	
Baseline Total Cholesterol (mg/dl) Median	167	245	207	0.472
Baseline HDL Cholesterol (mg/dl) Median	35	46	44	0.835
Baseline Blood Glucose (mg/dl) Median	82	87	97	0.488
Baseline LDL Cholesterol (mg/dl) Median	96	136	125	0.581
Baseline Triglycerides (mg/dl) Median	162	178	144	0.161
W24 Total Cholesterol (mg/dl) Median	172	210	195	0.961
W24 HDL Cholesterol (mg/dl) Median	39	43	45	0.788
W24 Blood Glucose (mg/dl) Median	90	87	95	0.854
W24 LDL Cholesterol (mg/dl) Median	101	131	100	0.821
W24 Triglycerides (mg/dl) Median	120	151	166	0.514
Gender % (M/F)	73/27	47/53	25/75	

Figure 6.2 below shows the cycle threshold analysis of the allelic discrimination assay. There were no significant correlations with baseline lipid parameters for any of the genotypes, although median total cholesterol and LDL-C were higher at both the baseline and week 24 where at least one copy of the A allele was present, Figure 6.3 (A and D) below. Median TG values were higher at both the baseline and week 24 where at least one copy of the G allele was present, Figure 6.3 (E) below. A trend was associated with higher LDL-C levels (P=0.0817) in females with the A allele, within ABCA1 (rs2230806G/A), see Figure 6.4 below.

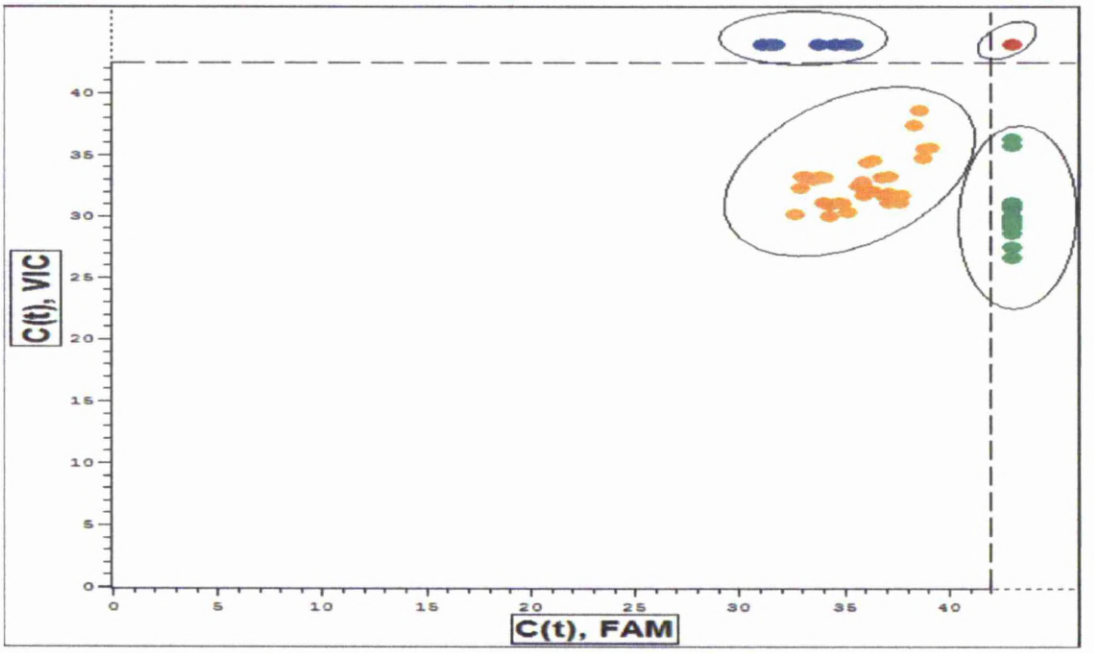
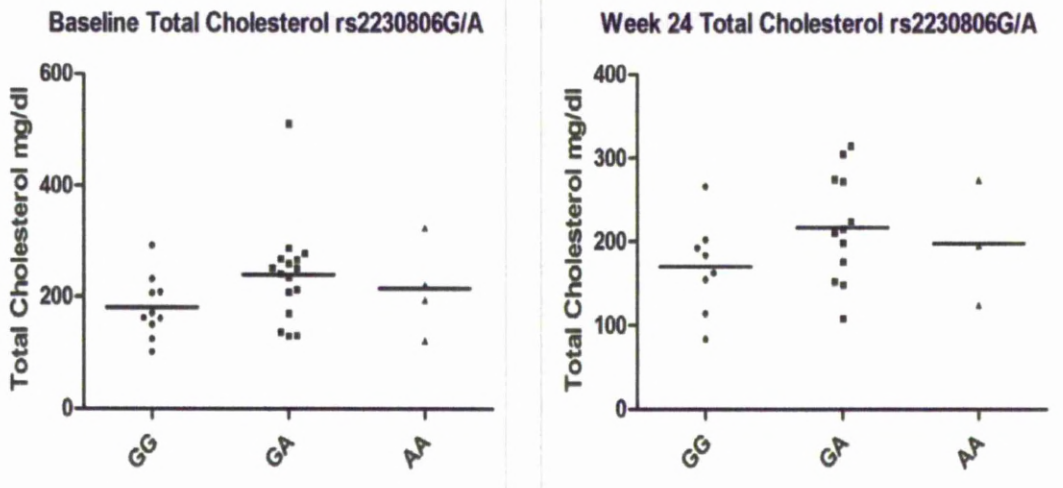
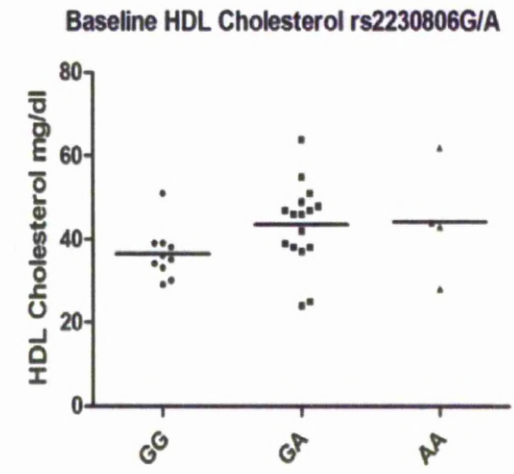


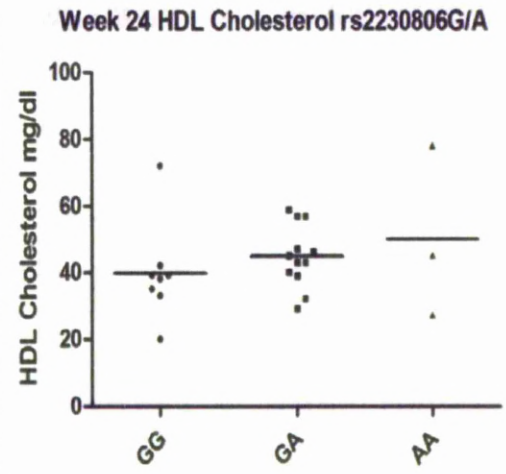
Figure 6.2: - ABCA1 SNP rs2230806G/A - Cycle threshold analysis of allelic discrimination assay by qPCR.

A

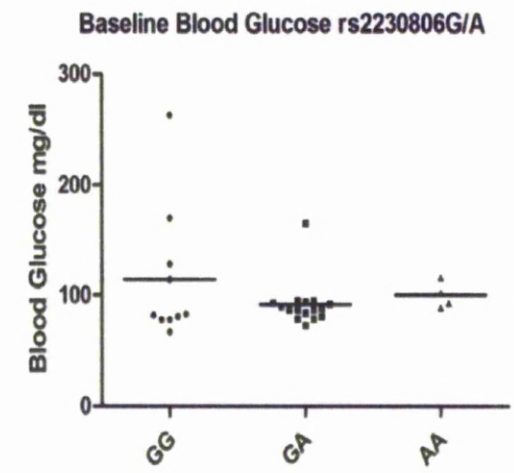


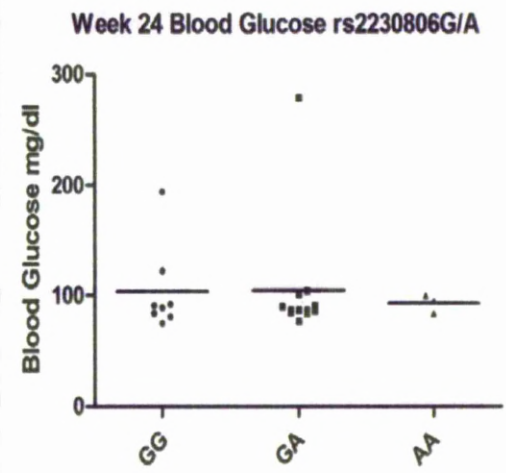
B


Baseline HDL Cholesterol rs2230806G/A
HDL Cholesterol mg/dl
0
20
40
60
80
GG
GA
AA

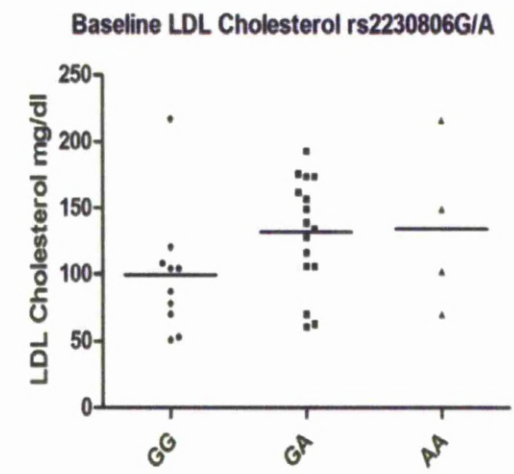

Week 24 HDL Cholesterol rs2230806G/A
HDL Cholesterol mg/dl
0
20
40
60
80
100
GG
GA
AA

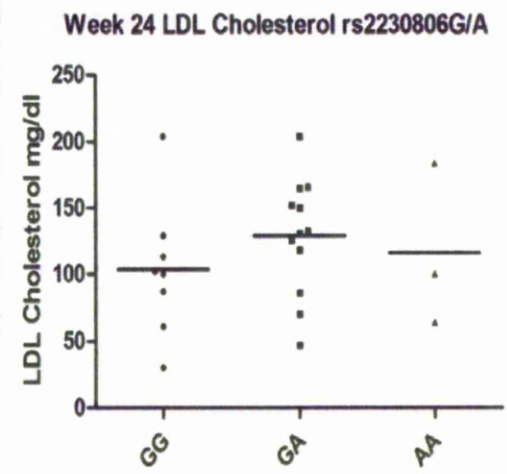
C


Baseline Blood Glucose rs2230806G/A
Blood Glucose mg/dl
0
100
200
300
GG
GA
AA


Week 24 Blood Glucose rs2230806G/A
Blood Glucose mg/dl
0
100
200
300
GG
GA
AA

D


Baseline LDL Cholesterol rs2230806G/A
LDL Cholesterol mg/dl
0
50
100
150
200
250
GG
GA
AA


Week 24 LDL Cholesterol rs2230806G/A
LDL Cholesterol mg/dl
0
50
100
150
200
250
GG
GA
AA

E

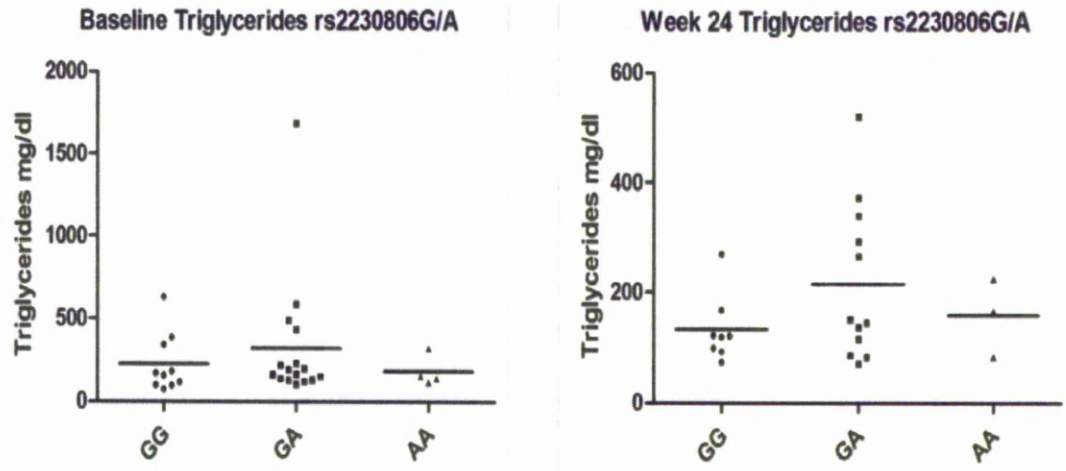


Figure 6.3: - ABCA1 SNP rs2230806G/A - Analysis of genotype influence on lipid parameters: A= Comparison of Baseline Total Cholesterol levels against Week 24 Total cholesterol levels (mg/dl); B= Comparison of Baseline HDL-C levels against Week 24 HDL-C levels (mg/dl); C= Comparison of Baseline Fasted Blood Glucose levels against Week 24 Fasted Blood Glucose levels (mg/dl); D= Comparison of Baseline LDL-C levels against Week 24 LDL-C levels (mg/dl); E= Comparison of Baseline TG levels against Week 24 TG levels (mg/dl) Median values denoted by black line.

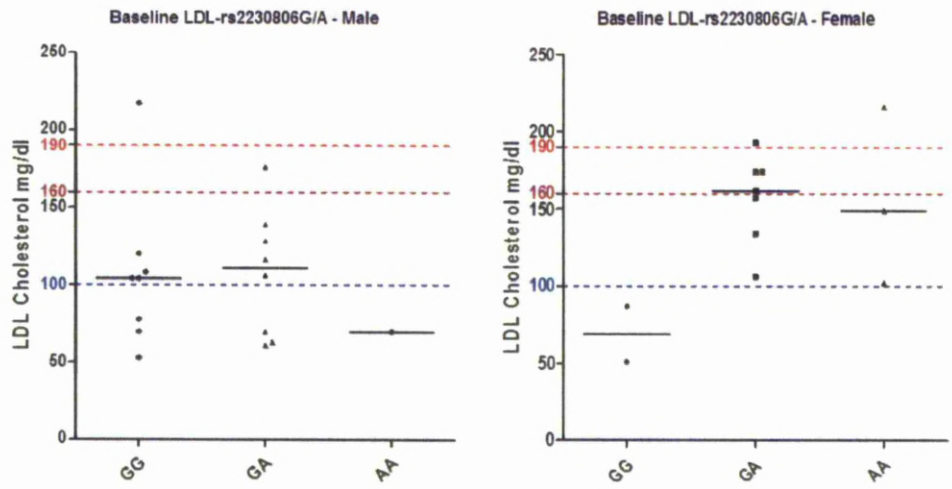


Figure 6.4: - ABCA1 SNP rs2230806G/A - Analysis of genotype influence on LDL-C parameters by Gender- Comparison of Baseline LDL-C levels against Week 24 LDL-C levels (mg/dl) Median values denoted by black line, red line denotes upper thresholds of lipid parameters whilst blue dotted line denotes upper limit of normal range, where shown.

6.5.4 LDLR Genotyping and Association with Metabolic Parameters

The distribution of the genotypes for the rs1529729C/T SNP within LDLR included 5 individuals with the CC genotype (3 male and 2 female), 13 heterozygotes with the CT genotype (5 male and 8 female) and 14 homozygous TT individuals (9 male and 5 female). Univariate analysis of the patient data did not confirm any significant associations between the patient genotype and the baseline lipid parameters, however, at week 24 a significant association between the TT genotype and triglyceride levels was observed (P=0.011), Table 6.4.

Table 6.4: Univariate analysis of LDLR rs1529729C/T SNP in Italian Patient Cohort on Metabolic Parameters (n=32)

	CC	CT	TT	P Value
Patient Numbers	5	13	14	
Baseline Total Cholesterol (mg/dl) Median	220	246	171	0.415
Baseline HDL Cholesterol (mg/dl) Median	42	43	39	0.556
Baseline Blood Glucose (mg/dl) Median	88	95	85	0.607
Baseline LDL Cholesterol (mg/dl) Median	139	118	103	0.993
Baseline Triglycerides (mg/dl) Median	166	195	150	0.22
W24 Total Cholesterol (mg/dl) Median	241	195	192	0.6
W24 HDL Cholesterol (mg/dl) Median	46	42	39	0.394
W24 Blood Glucose (mg/dl) Median	96	89	89	0.615
W24 LDL Cholesterol (mg/dl) Median	142	124	102	0.941
W24 Triglycerides (mg/dl) Median	261	216	116	0.011
Gender % (M/F)	60/40	39/61	64/36	

Figure 6.5 below shows the cycle threshold analysis of the allelic discrimination assay. Figure 6.6, below, shows the distribution of baseline lipid parameters contrasted with the corresponding week 24 parameters by genotype with median parameter values highlighted within each scatter plot.

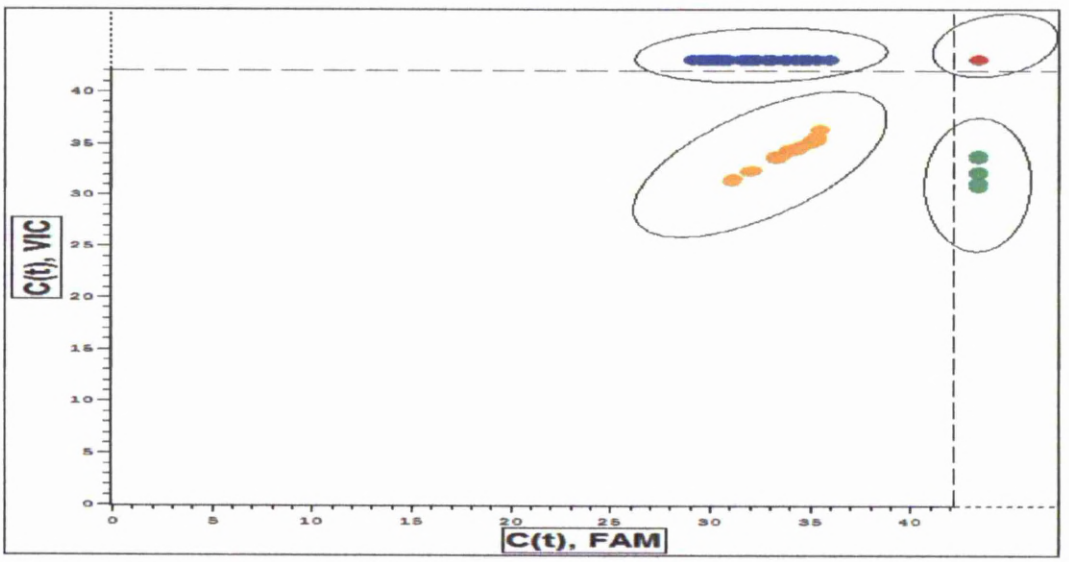
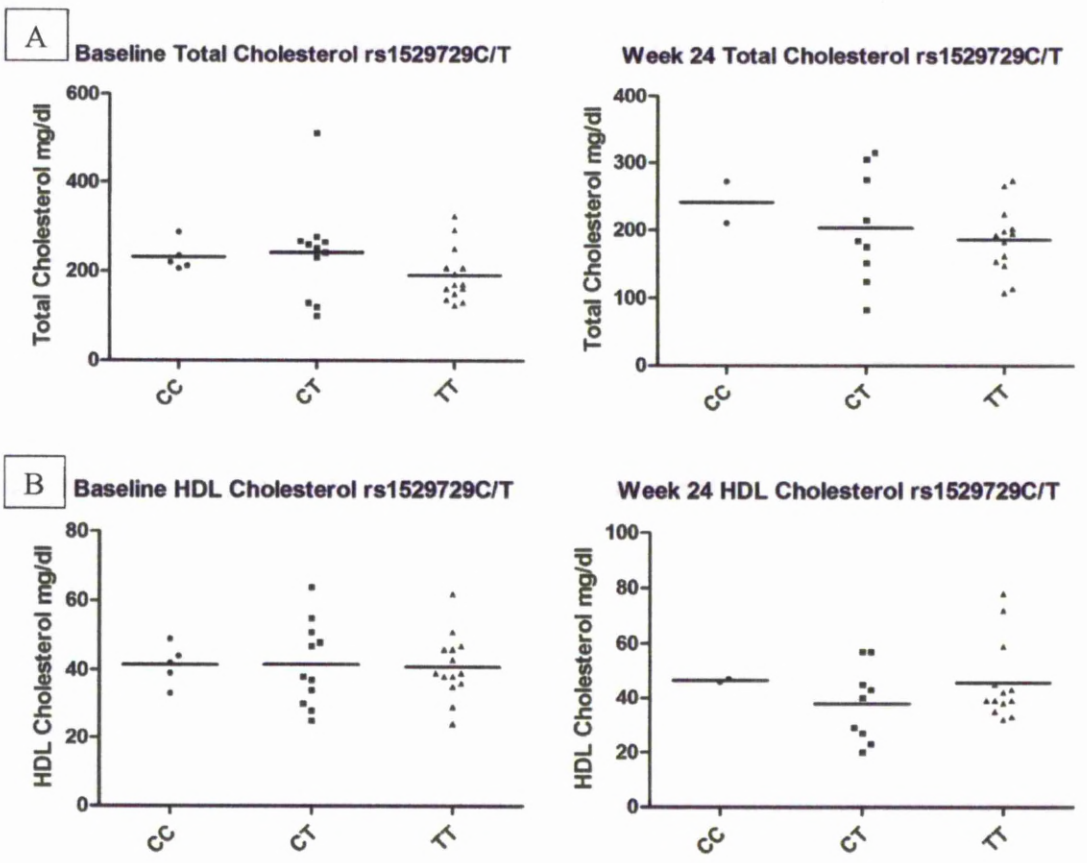


Figure 6.5: LDLR rs1529729C/T - Cycle threshold analysis of allelic discrimination assay by qPCR.



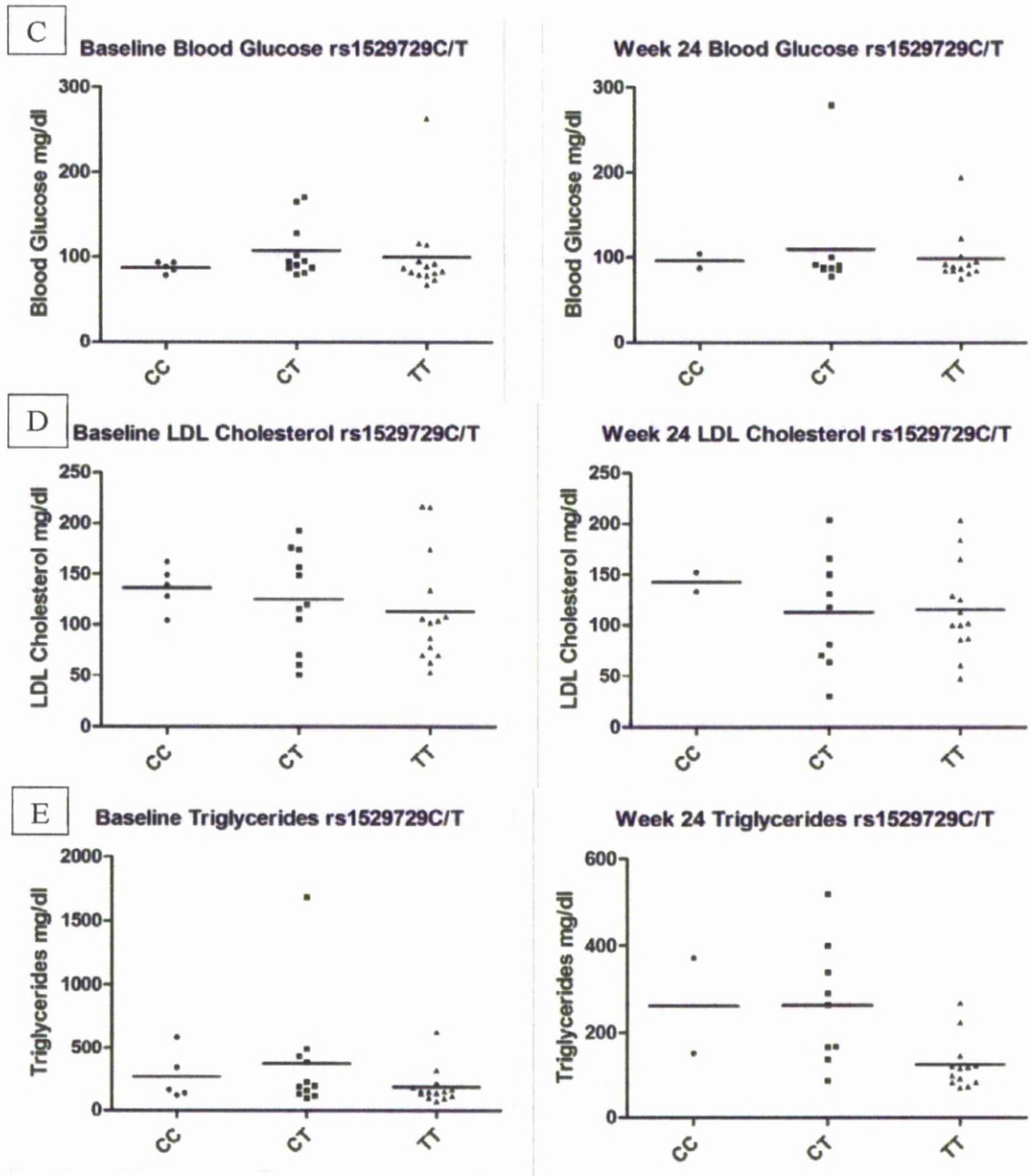


Figure 6.6: - LDLR SNP rs1529729C/T - Analysis of genotype influence on lipid parameters: A= Comparison of Baseline Total Cholesterol levels against Week 24 Total cholesterol levels (mg/dl); B= Comparison of Baseline HDL-C levels against Week 24 HDL-C levels (mg/dl); C= Comparison of Baseline Fasted Blood Glucose levels against Week 24 Fasted Blood Glucose levels (mg/dl); D= Comparison of Baseline LDL-C levels against Week 24 LDL-C levels (mg/dl); E= Comparison of Baseline TG levels against Week 24 TG levels (mg/dl) Median values denoted by black line.

The distribution of the genotypes for the rs2228671C/T SNP within LDLR included 30 individuals with the CC genotype (16 males and 14 females) and 2 heterozygotes

with the CT genotype (1 male and 1 female). Figure 6.7 below shows the cycle threshold analysis of the allelic discrimination assay. Univariate analysis of the patient data did not confirm any significant associations between the patient genotype and the baseline lipid parameters, however, a trend was observed between the wild type homozygous CC genotype and baseline triglyceride levels (P=0.059), Table 6.5. This should be interpreted with caution as the homozygous rare genotype TT was not represented in the patient cohort. There were no significant associations in the univariate analysis of the CC and CT genotypes and week 24 lipid levels, Table 6.5.

Table 6.5: Univariate analysis of LDLR rs2228671C/T SNP in Italian Patient Cohort on Metabolic Parameters (n=32)

	CC	CT	TT	P Value
Patient Numbers	30	2	0	
Baseline Total Cholesterol (mg/dl) Median	208	239	NA	0.187
Baseline HDL Cholesterol (mg/dl) Median	39	48	NA	0.139
Baseline Blood Glucose (mg/dl) Median	89	90	NA	0.379
Baseline LDL Cholesterol (mg/dl) Median	106	166	NA	0.193
Baseline Triglycerides (mg/dl) Median	166	124	NA	0.059
W24 Total Cholesterol (mg/dl) Median	197	175	NA	0.813
W24 HDL Cholesterol (mg/dl) Median	43	40	NA	0.928
W24 Blood Glucose (mg/dl) Median	89	87	NA	0.993
W24 LDL Cholesterol (mg/dl) Median	119	118	NA	0.971
W24 Triglycerides (mg/dl) Median	141	86	NA	0.484
Gender % (M/F)	53/47	50/50	0/0	

Figure 6.8, below, shows the distribution of baseline lipid parameters for rs2228671C/T contrasted with the corresponding week 24 parameters by genotype with median parameter values highlighted within each scatter plot.

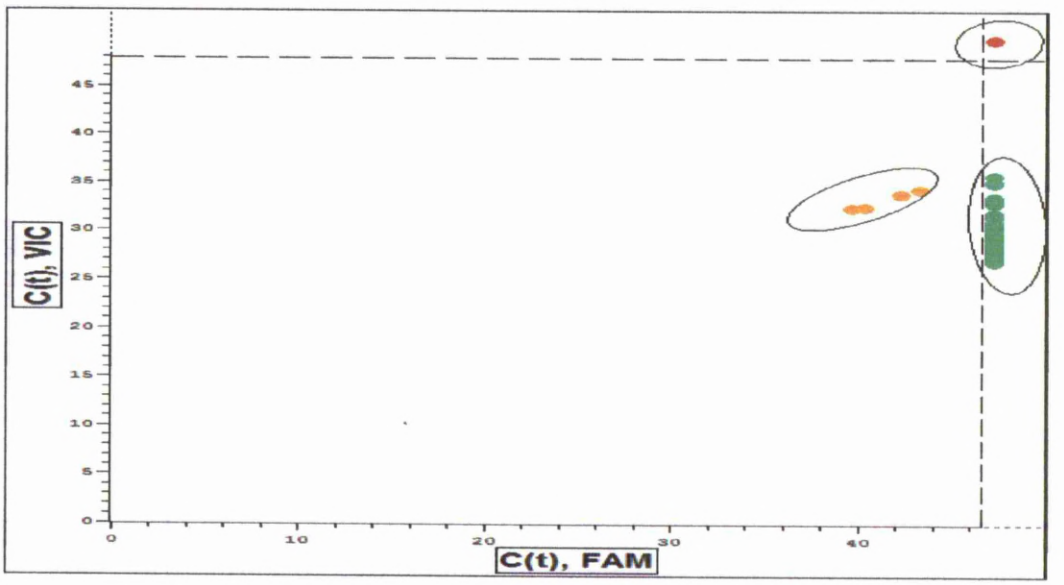
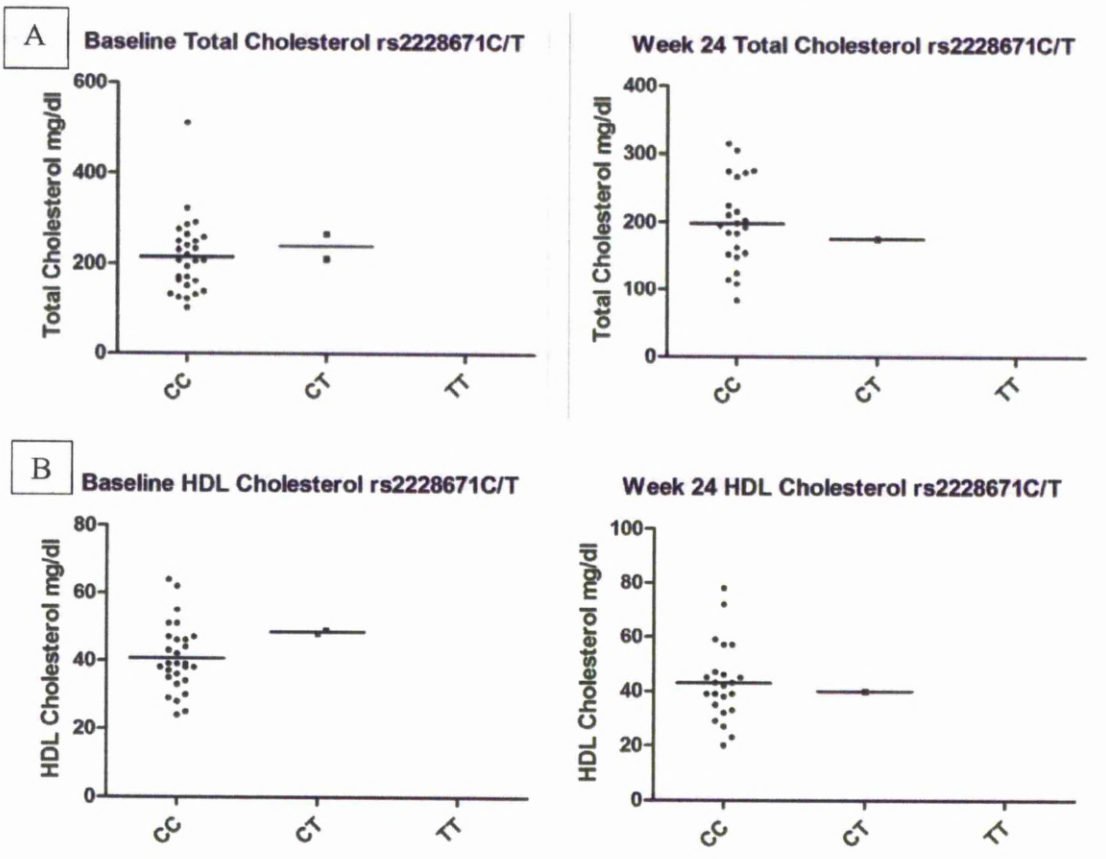


Figure 6.7: LDLR rs2228671C/T - Cycle threshold analysis of allelic discrimination assay by qPCR.



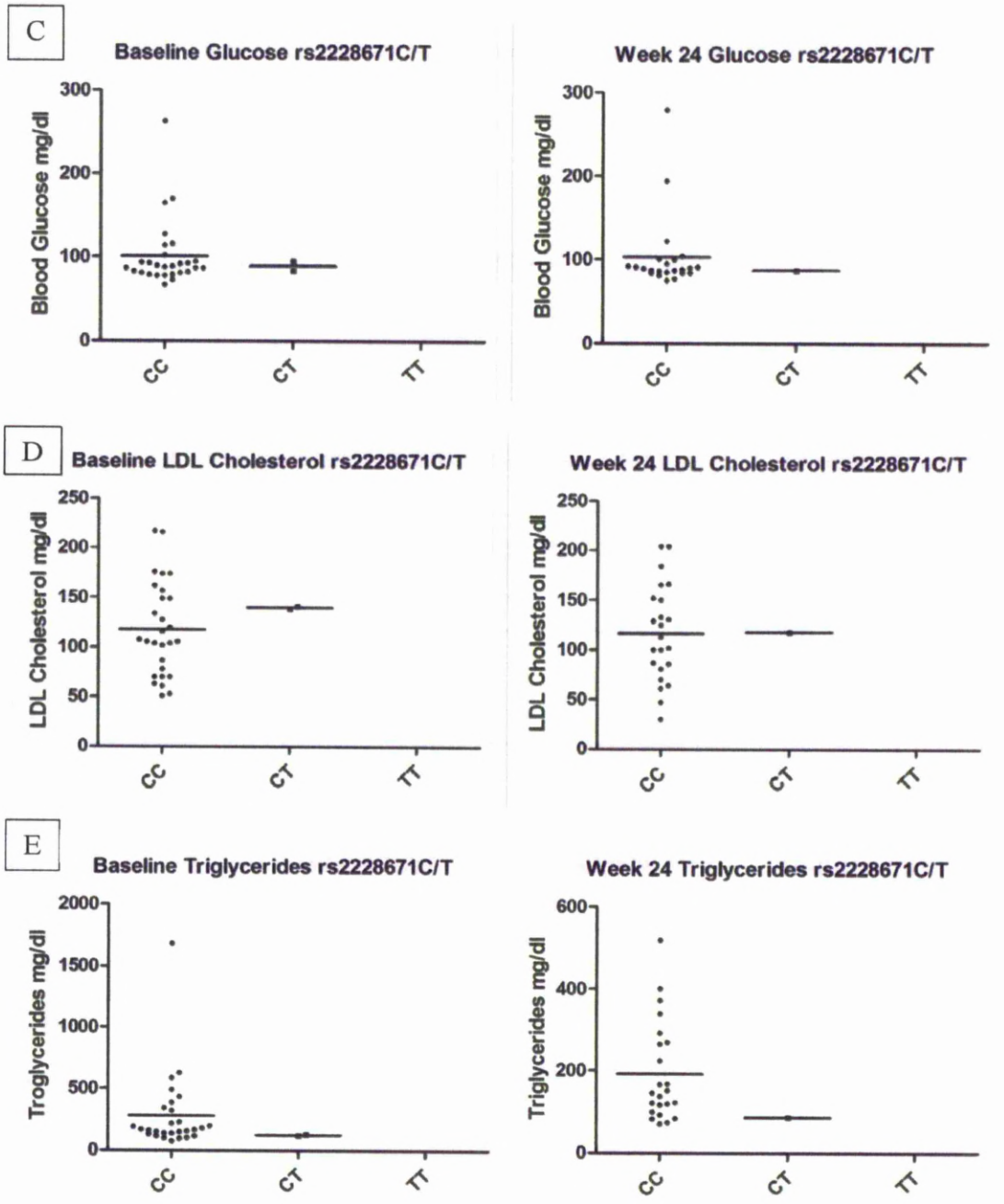


Figure 6.8: - LDLR SNP rs2228671C/T - Analysis of genotype influence on lipid parameters: A= Comparison of Baseline Total Cholesterol levels against Week 24 Total cholesterol levels (mg/dl); B= Comparison of Baseline HDL-C levels against Week 24 HDL-C levels (mg/dl); C= Comparison of Baseline Fasted Blood Glucose levels against Week 24 Fasted Blood Glucose levels (mg/dl); D= Comparison of Baseline LDL-C levels against Week 24 LDL-C levels (mg/dl); E= Comparison of Baseline TG levels against Week 24 TG levels (mg/dl) Median values denoted by black line.

6.5.5 SCARB1 Genotyping and Association with Metabolic Parameters

The distribution of the genotypes for the rs2278986C/T SNP within SCARB1 included 2 individuals with the CC genotype (2 females) and 22 heterozygotes with the CT genotype (13 males and 9 females) and 8 homozygous TT individuals (4 males and 4 females). Figure 6.9 below shows the cycle threshold analysis of the allelic discrimination assay, endpoint analysis is also shown in Figure 6.10 for validation purposes. Univariate analysis confirmed that there were no significant correlations with baseline lipid parameters and week 24 lipid for any of the genotypes, although a trend was associated with the homozygous wild type CC genotype and week 24 HDL cholesterol levels (P=0.098) , Table 6.6. This should be interpreted with caution as there were only 2 females in this genotype group of the patient cohort and the findings would need to be validated in a larger cohort to prove this association.

Table 6.6: Univariate analysis of SCARB1 rs2278986C/T SNP in Italian Patient Cohort on Metabolic Parameters (n=32)

	CC	CT	TT	P Value
Patient Numbers	2	22	8	
Baseline Total Cholesterol (mg/dl) Median	197	208	216	0.155
Baseline HDL Cholesterol (mg/dl) Median	45	39	40	0.529
Baseline Blood Glucose (mg/dl) Median	87	87	104	0.695
Baseline LDL Cholesterol (mg/dl) Median	124	107	130	0.867
Baseline Triglycerides (mg/dl) Median	140	175	158	0.183
W24 Total Cholesterol (mg/dl) Median	197	197	157	0.597
W24 HDL Cholesterol (mg/dl) Median	60	39	35	0.098
W24 Blood Glucose (mg/dl) Median	90	88	89	0.772
W24 LDL Cholesterol (mg/dl) Median	110	121	86	0.53
W24 Triglycerides (mg/dl) Median	137	129	195	0.336
Gender % (M/F)	0/100	59/41	50/50	

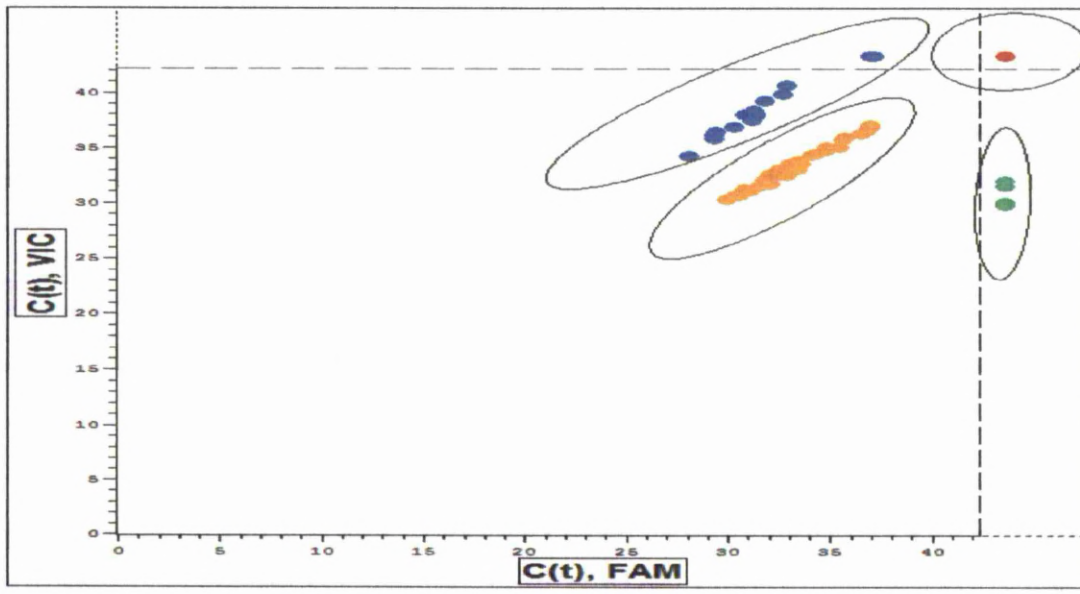


Figure 6.9: SCARB1 rs2278986C/T - Cycle threshold analysis of allelic discrimination assay by qPCR.

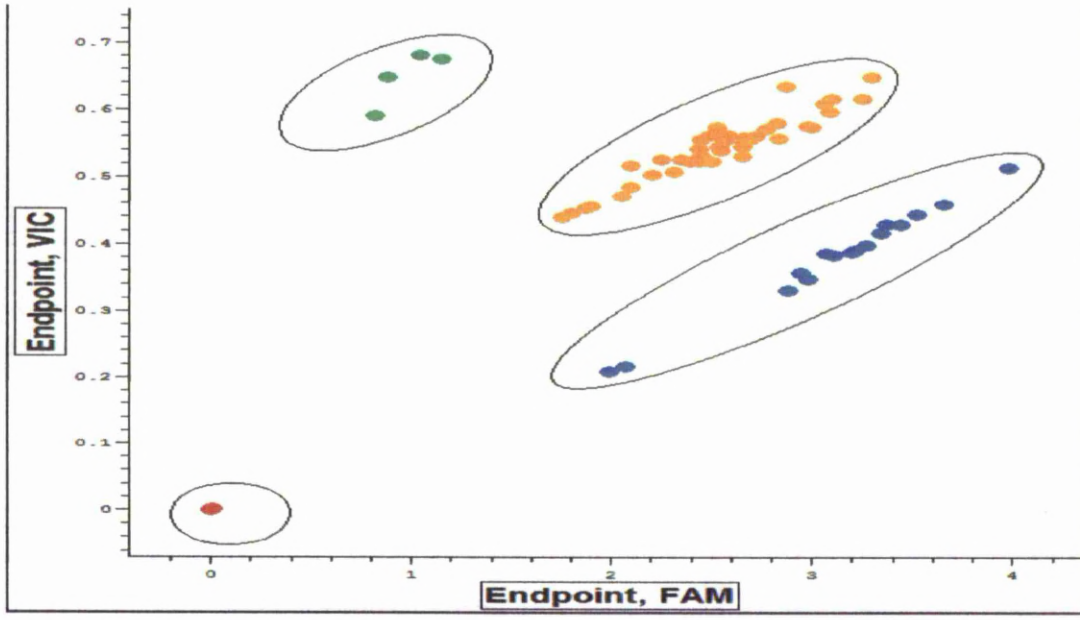
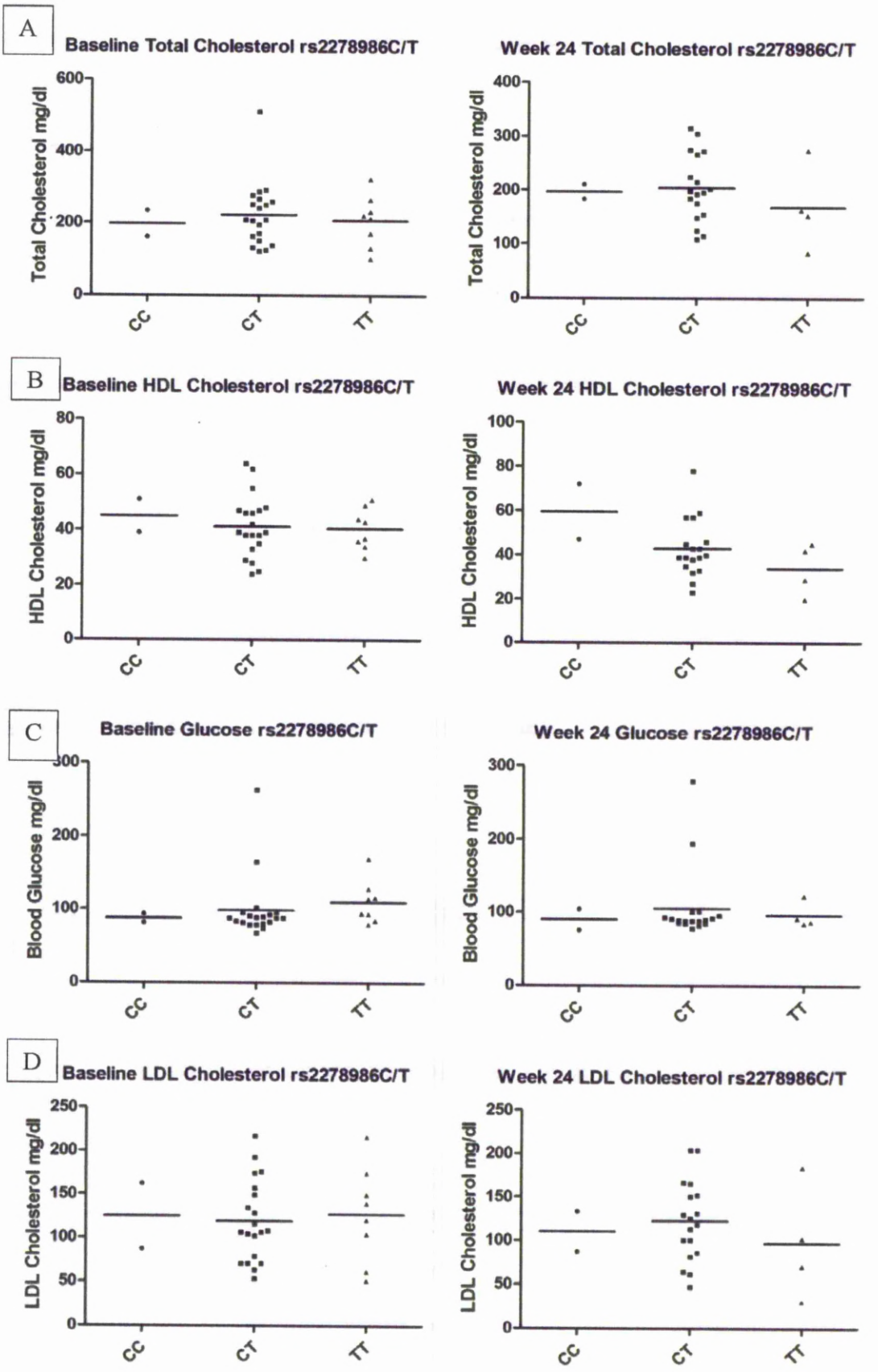


Figure 6.10: SCARB1 rs2278986C/T - Endpoint analysis of allelic discrimination assay by qPCR.

Figure 6.11, below, shows the distribution of baseline lipid parameters for rs2278986C/T contrasted with the corresponding week 24 parameters by genotype with median parameter values highlighted within each scatter plot.


A
Baseline Total Cholesterol rs2278986C/T
Total Cholesterol mg/dl
600
400
200
0
CC
CT
TT
Week 24 Total Cholesterol rs2278986C/T
Total Cholesterol mg/dl
400
300
200
100
0
CC
CT
TT
B
Baseline HDL Cholesterol rs2278986C/T
HDL Cholesterol mg/dl
80
60
40
20
0
CC
CT
TT
Week 24 HDL Cholesterol rs2278986C/T
HDL Cholesterol mg/dl
100
80
60
40
20
0
CC
CT
TT
C
Baseline Glucose rs2278986C/T
Blood Glucose mg/dl
300
200
100
0
CC
CT
TT
Week 24 Glucose rs2278986C/T
Blood Glucose mg/dl
300
200
100
0
CC
CT
TT
D
Baseline LDL Cholesterol rs2278986C/T
LDL Cholesterol mg/dl
250
200
150
100
50
0
CC
CT
TT
Week 24 LDL Cholesterol rs2278986C/T
LDL Cholesterol mg/dl
250
200
150
100
50
0
CC
CT
TT

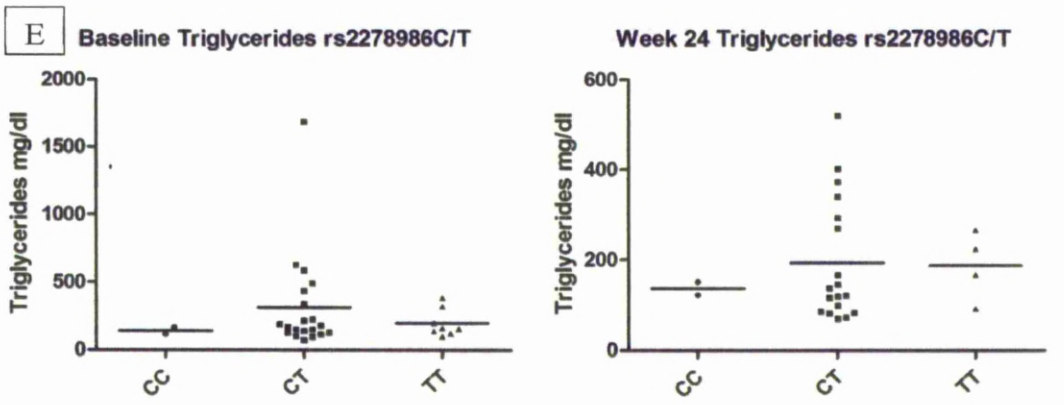


Figure 6.11: - SCARB1 SNP rs2278986C/T - Analysis of genotype influence on lipid parameters: A= Comparison of Baseline Total Cholesterol levels against Week 24 Total cholesterol levels (mg/dl); B= Comparison of Baseline HDL-C levels against Week 24 HDL-C levels (mg/dl); C= Comparison of Baseline Fasted Blood Glucose levels against Week 24 Fasted Blood Glucose levels (mg/dl); D= Comparison of Baseline LDL-C levels against Week 24 LDL-C levels (mg/dl); E= Comparison of Baseline TG levels against Week 24 TG levels (mg/dl) Median values denoted by black line.

6.5.6 ABCG1 Genotyping and Association with Metabolic Parameters

The distribution of the genotypes for the rs1893590A/C SNP within ABCG1 included 18 individuals with the AA genotype (9 male and 9 female), 13 heterozygotes with the AC genotype (7 male and 6 female) and 1 homozygous CC individual (1 male). Figure 6.12 below shows the cycle threshold analysis of the allelic discrimination assay. There were no significant associations in the univariate analysis of the genotypes with baseline and week 24 lipid parameters, Table 6.7, however, a trend was observed with the homozygous CC genotype and baseline triglyceride levels (P=0.096). This association should be interpreted with caution as there was only one patient in the cohort with the CC genotype. Further validation in a larger cohort would be necessary to establish the significance of this association. Figure 6.13, below, shows the distribution of baseline lipid parameters for rs1893590A/C compared to the corresponding week 24 parameters by genotype with median parameter values highlighted within each scatter plot.

Table 6.7: Univariate analysis of ABCG1 rs1893590A/C SNP in Italian Patient Cohort on Metabolic Parameters (n=32)

	AA	AC	CC	P Value
Patient Numbers	18	13	1	
Baseline Total Cholesterol (mg/dl) Median	167	245	207	0.377
Baseline HDL Cholesterol (mg/dl) Median	35	46	44	0.412
Baseline Blood Glucose (mg/dl) Median	82	87	97	0.622
Baseline LDL Cholesterol (mg/dl) Median	96	136	125	0.584
Baseline Triglycerides (mg/dl) Median	162	178	144	0.096
W24 Total Cholesterol (mg/dl) Median	172	210	195	0.7
W24 HDL Cholesterol (mg/dl) Median	39	43	45	0.87
W24 Blood Glucose (mg/dl) Median	90	87	95	0.702
W24 LDL Cholesterol (mg/dl) Median	101	131	100	0.531
W24 Triglycerides (mg/dl) Median	120	151	166	0.453
Gender % (M/F)	50/50	54/46	100/0	

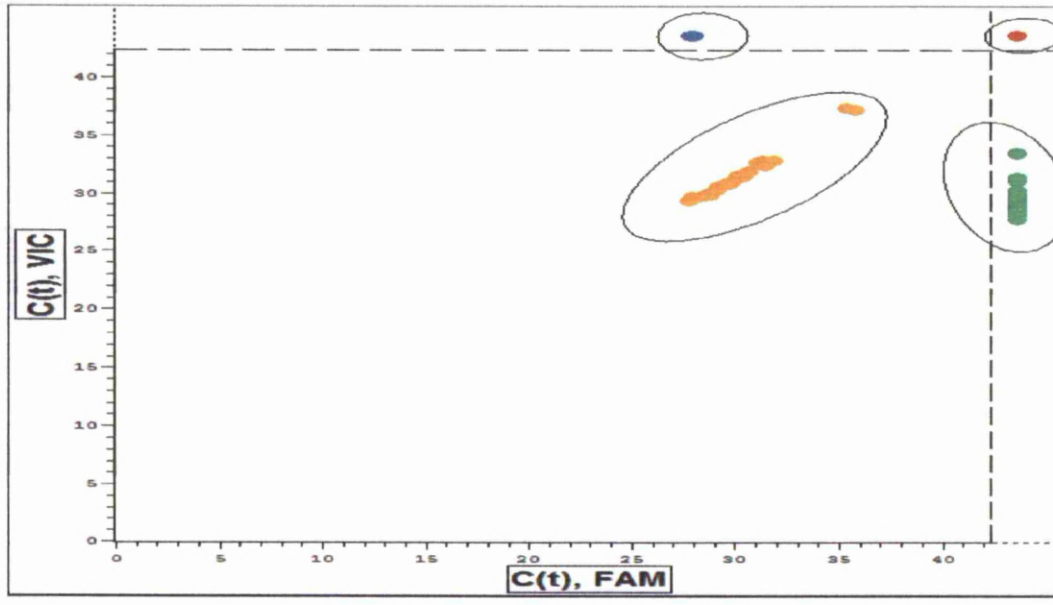
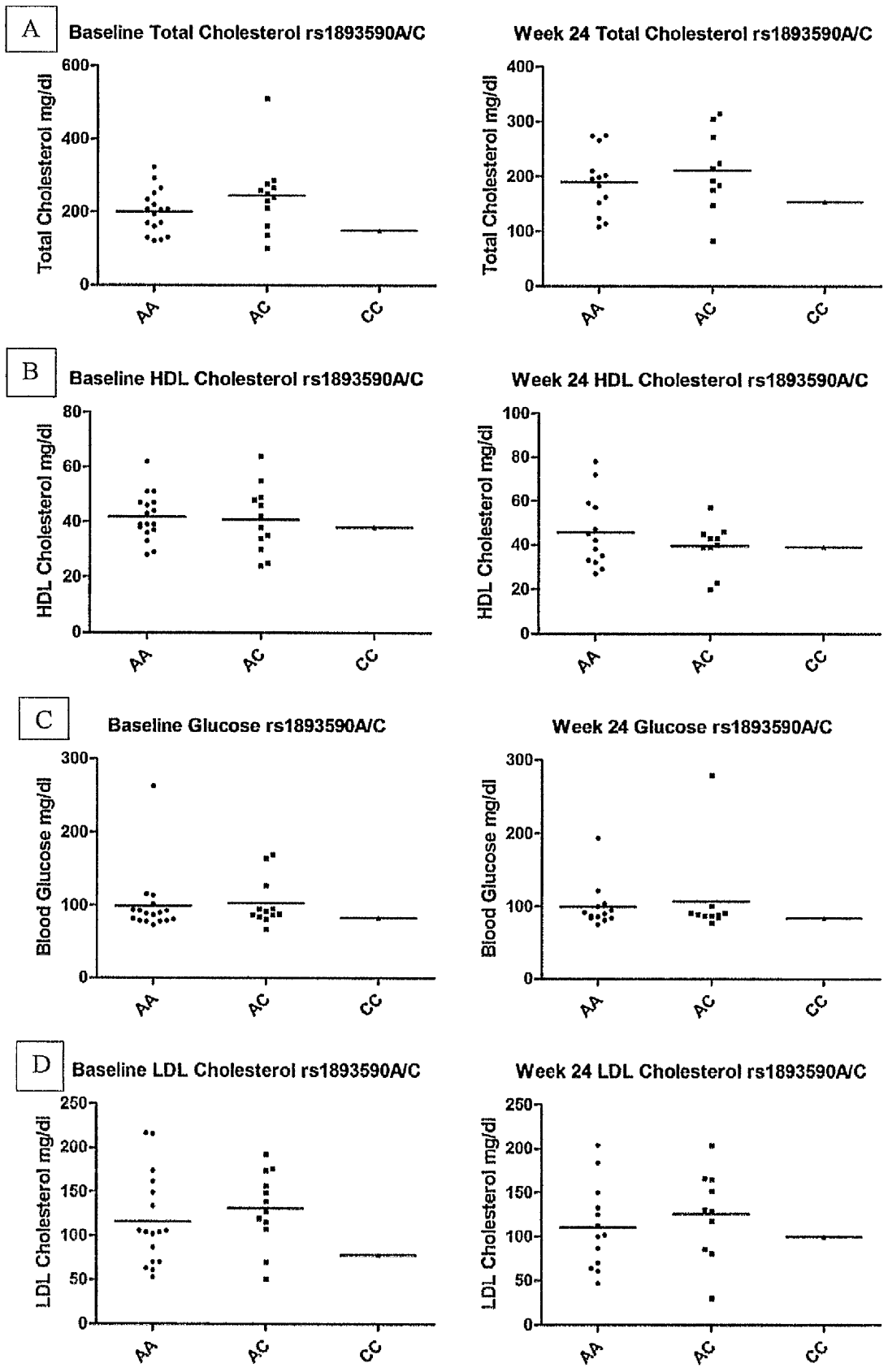


Figure 6.12: ABCG1 rs1893590A/C - Cycle threshold analysis of allelic discrimination assay by qPCR


A
Baseline Total Cholesterol rs1893590A/C
Total Cholesterol mg/dl
600
400
200
0
AA
AC
CC
Week 24 Total Cholesterol rs1893590A/C
Total Cholesterol mg/dl
400
300
200
100
0
AA
AC
CC
B
Baseline HDL Cholesterol rs1893590A/C
HDL Cholesterol mg/dl
80
60
40
20
0
AA
AC
CC
Week 24 HDL Cholesterol rs1893590A/C
HDL Cholesterol mg/dl
100
80
60
40
20
0
AA
AC
CC
C
Baseline Glucose rs1893590A/C
Blood Glucose mg/dl
300
200
100
0
AA
AC
CC
Week 24 Glucose rs1893590A/C
Blood Glucose mg/dl
300
200
100
0
AA
AC
CC
D
Baseline LDL Cholesterol rs1893590A/C
LDL Cholesterol mg/dl
250
200
150
100
50
0
AA
AC
CC
Week 24 LDL Cholesterol rs1893590A/C
LDL Cholesterol mg/dl
250
200
150
100
50
0
AA
AC
CC

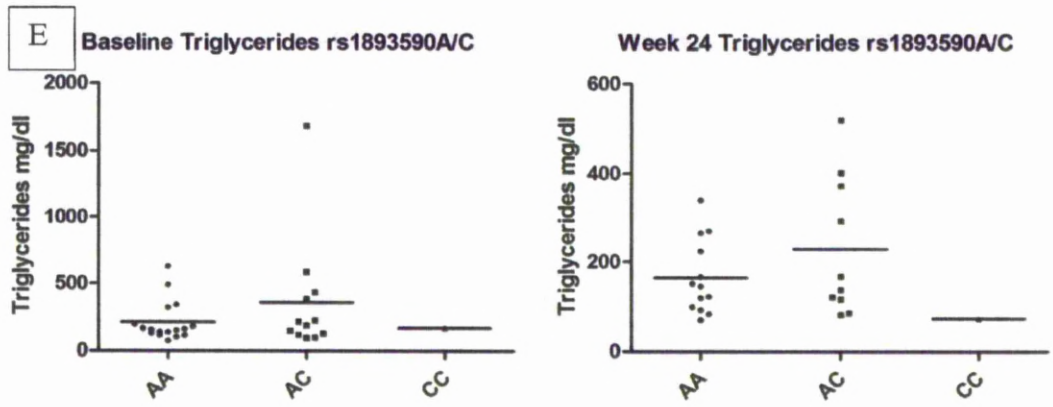


Figure 6.13: - ABCG1 SNP rs1893590A/C - Analysis of genotype influence on lipid parameters: A= Comparison of Baseline Total Cholesterol levels against Week 24 Total cholesterol levels (mg/dl); B= Comparison of Baseline HDL-C levels against Week 24 HDL-C levels (mg/dl); C= Comparison of Baseline Fasted Blood Glucose levels against Week 24 Fasted Blood Glucose levels (mg/dl); D= Comparison of Baseline LDL-C levels against Week 24 LDL-C levels (mg/dl); E= Comparison of Baseline TG levels against Week 24 TG levels (mg/dl) Median values denoted by black line.

6.5.7 ABCG8 Genotyping and Association with Metabolic Parameters

The distribution of the genotypes for the rs6544713C/T SNP within ABCG8 included 13 individuals with the CC genotype (6 male and 7 female), 14 heterozygotes with the CT genotype (6 male and 8 female) and 5 homozygous TT individuals (5 male). Figure 6.14 below shows the cycle threshold analysis of the allelic discrimination assay. There were no significant associations in the univariate analysis with baseline lipid parameters and week 24 lipid levels for any of the genotypes. Trends were observed, however, between the T allele and increased baseline HDL (P=0.095) and lower baseline triglycerides with the homozygous rare TT genotype, (P=0.056). At week 24 a trend was observed increased blood glucose levels and the rare homozygous TT genotype, (P=0.095), Table 6.8.

Table 6.8: Univariate analysis of ABCG8 rs6544713C/T SNP in Italian Patient Cohort on Metabolic Parameters (n=32)

	CC	CT	TT	P Value
Patient Numbers	13	14	5	
Baseline Total Cholesterol (mg/dl) Median	167	245	207	0.538
Baseline HDL Cholesterol (mg/dl) Median	35	46	44	0.095
Baseline Blood Glucose (mg/dl) Median	82	87	97	0.508
Baseline LDL Cholesterol (mg/dl) Median	96	136	125	0.472
Baseline Triglycerides (mg/dl) Median	162	178	144	0.056
W24 Total Cholesterol (mg/dl) Median	172	210	195	0.892
W24 HDL Cholesterol (mg/dl) Median	39	43	45	0.441
W24 Blood Glucose (mg/dl) Median	90	87	95	0.095
W24 LDL Cholesterol (mg/dl) Median	101	131	100	0.751
W24 Triglycerides (mg/dl) Median	120	151	166	0.246
Gender % (M/F)	46/54	43/57	100/0	

Figure 6.15, below, shows the distribution of baseline lipid parameters for rs6544713C/T contrasted with the corresponding week 24 parameters by genotype with median parameter values highlighted within each scatter plot.

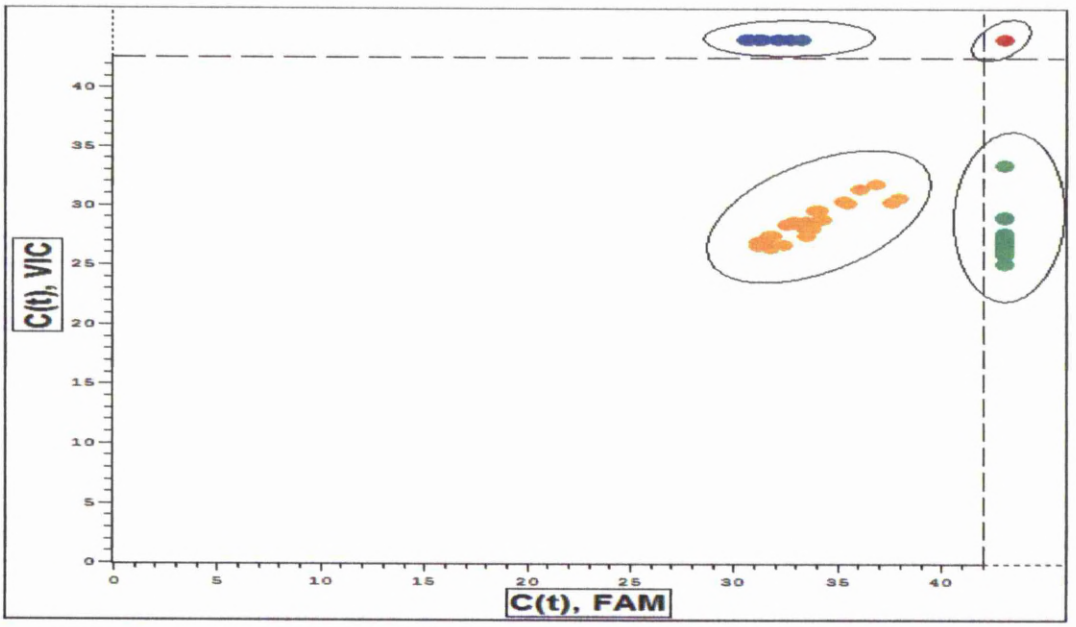
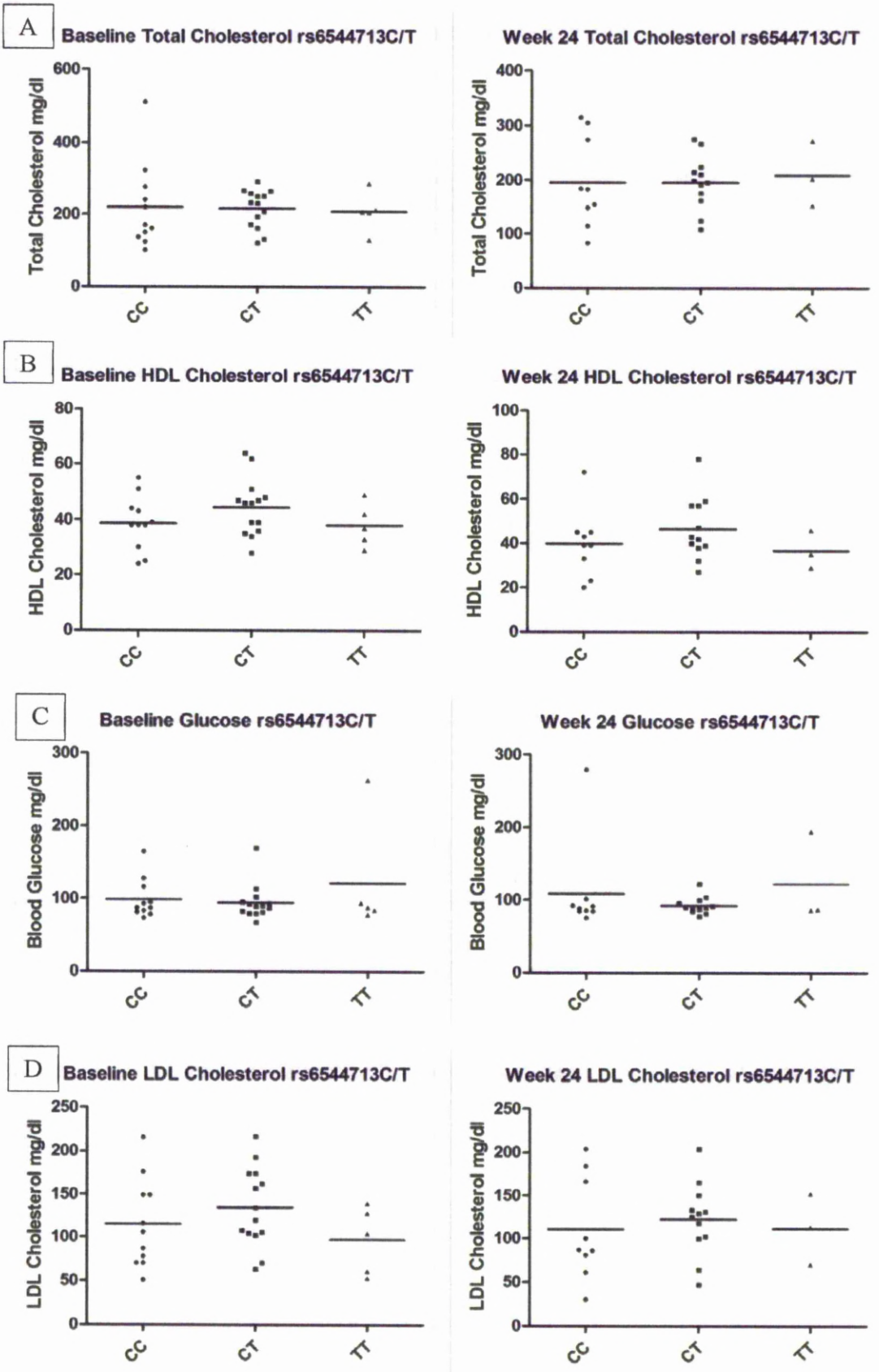


Figure 6.14: ABCG8 rs6544713C/T - Cycle threshold analysis of allelic discrimination assay by qPCR.


A
Baseline Total Cholesterol rs6544713C/T
Total Cholesterol mg/dl
600
400
200
0
CC
CT
TT
Week 24 Total Cholesterol rs6544713C/T
Total Cholesterol mg/dl
400
300
200
100
0
CC
CT
TT
B
Baseline HDL Cholesterol rs6544713C/T
HDL Cholesterol mg/dl
80
60
40
20
0
CC
CT
TT
Week 24 HDL Cholesterol rs6544713C/T
HDL Cholesterol mg/dl
100
80
60
40
20
0
CC
CT
TT
C
Baseline Glucose rs6544713C/T
Blood Glucose mg/dl
300
200
100
0
CC
CT
TT
Week 24 Glucose rs6544713C/T
Blood Glucose mg/dl
300
200
100
0
CC
CT
TT
D
Baseline LDL Cholesterol rs6544713C/T
LDL Cholesterol mg/dl
250
200
150
100
50
0
CC
CT
TT
Week 24 LDL Cholesterol rs6544713C/T
LDL Cholesterol mg/dl
250
200
150
100
50
0
CC
CT
TT

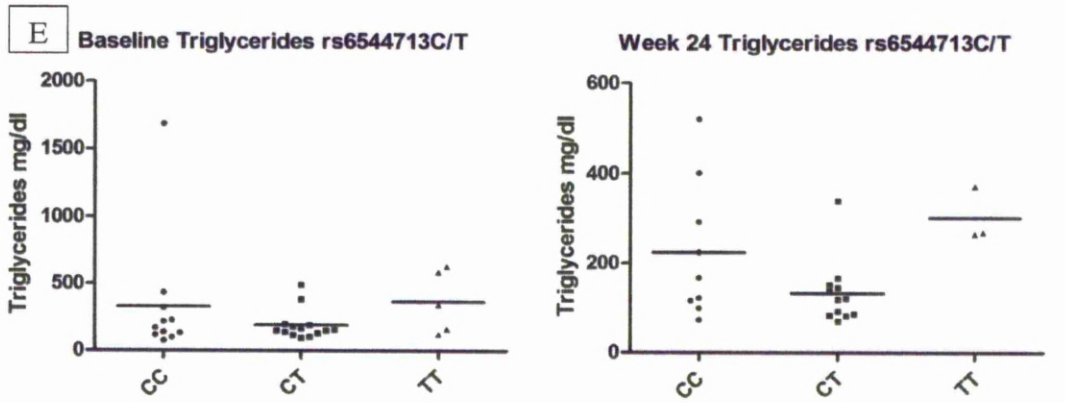


Figure 6.15: - ABCG8 SNP rs6544713C/T - Analysis of genotype influence on lipid parameters: A= Comparison of Baseline Total Cholesterol levels against Week 24 Total cholesterol levels (mg/dl); B= Comparison of Baseline HDL-C levels against Week 24 HDL-C levels (mg/dl); C= Comparison of Baseline Fasted Blood Glucose levels against Week 24 Fasted Blood Glucose levels (mg/dl); D= Comparison of Baseline LDL-C levels against Week 24 LDL-C levels (mg/dl); E= Comparison of Baseline TG levels against Week 24 TG levels (mg/dl) Median values denoted by black line.

6.5.8 ApoB Genotyping and Association with Metabolic Parameters

The distribution of the genotypes for the rs693A/G SNP within ApoB included 4 individuals with the AA genotype (1 male and 3 female), 15 heterozygotes with the AG genotype (9 male and 6 female) and 13 homozygous GG individuals (7 male and 6 female). Figure 6.16 below shows the cycle threshold analysis of the rs693A/G allelic discrimination assay. There were no significant correlations with baseline lipid parameters and week 24 lipid levels in the univariate analysis for any of the genotypes, although a trend was observed between baseline triglyceride levels and the homozygous GG genotype (P=0.07), Table 6.9.

Table 6.9: Univariate analysis of ApoB rs693A/G SNP in Italian Patient Cohort on Metabolic Parameters (n=32)

	AA	AG	GG	P Value
Patient Numbers	4	15	13	
Baseline Total Cholesterol (mg/dl) Median	167	245	207	0.995
Baseline HDL Cholesterol (mg/dl) Median	35	46	44	0.493
Baseline Blood Glucose (mg/dl) Median	82	87	97	0.92
Baseline LDL Cholesterol (mg/dl) Median	96	136	125	0.389
Baseline Triglycerides (mg/dl) Median	162	178	144	0.07
W24 Total Cholesterol (mg/dl) Median	172	210	195	0.476
W24 HDL Cholesterol (mg/dl) Median	39	43	45	0.777
W24 Blood Glucose (mg/dl) Median	90	87	95	0.823
W24 LDL Cholesterol (mg/dl) Median	101	131	100	0.602
W24 Triglycerides (mg/dl) Median	120	151	166	0.215
Gender % (M/F)	25/75	60/40	54/46	

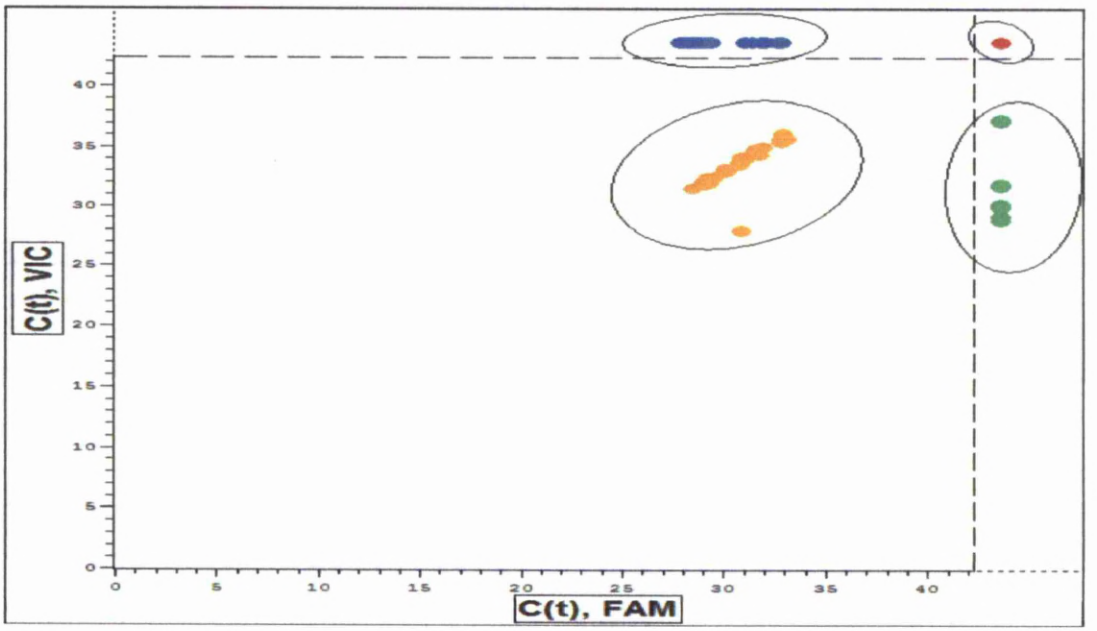
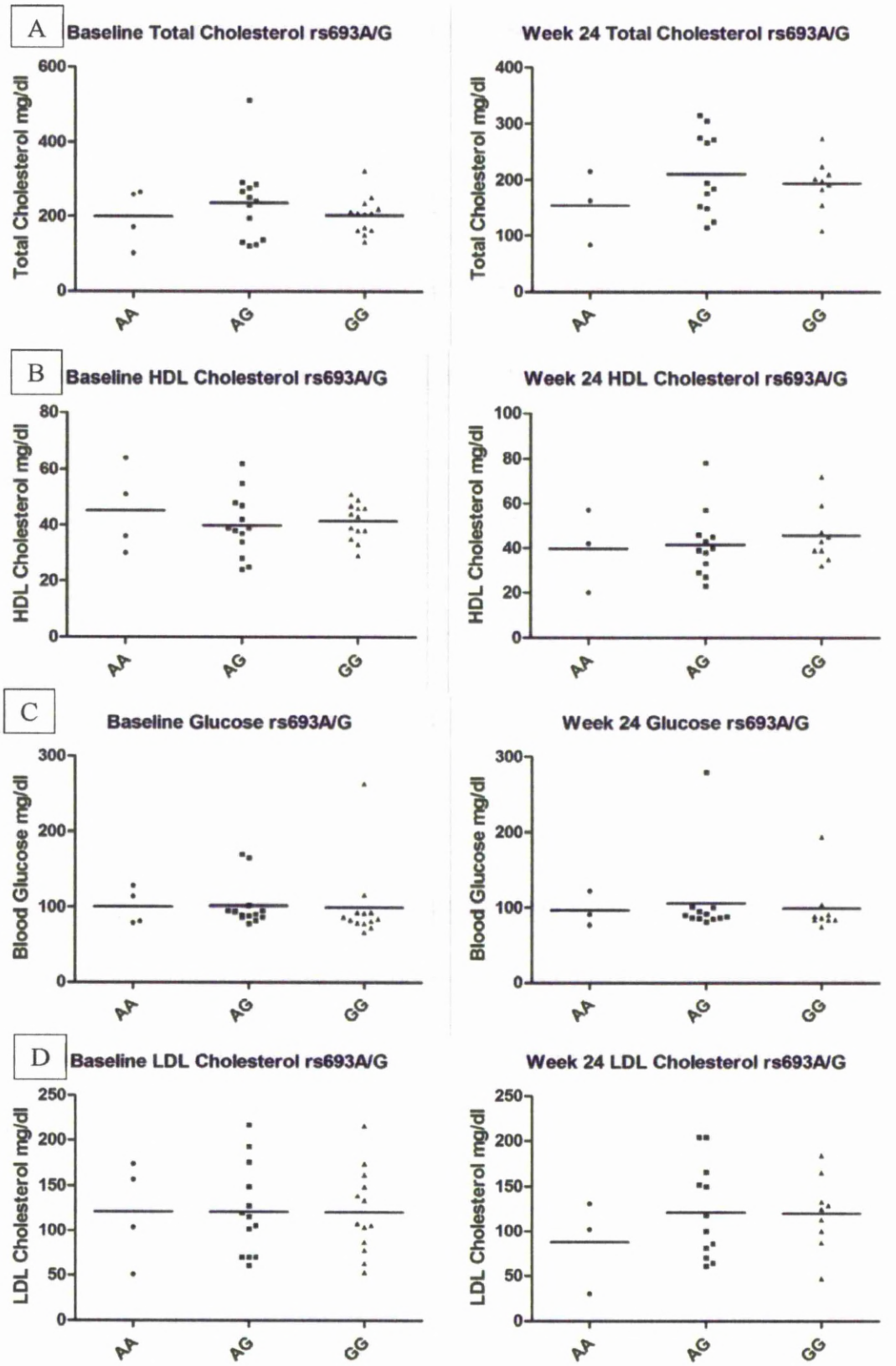


Figure 6.16: ApoB rs693A/G - Cycle threshold analysis of allelic discrimination assay by qPCR.

Figure 6.17, below, shows the distribution of baseline lipid parameters for rs693A/G compared to the corresponding week 24 parameters by genotype with median parameter values highlighted within each scatter plot.


A
Baseline Total Cholesterol rs693A/G
Total Cholesterol mg/dl
600
400
200
0
AA
AG
GG
Week 24 Total Cholesterol rs693A/G
Total Cholesterol mg/dl
400
300
200
100
0
AA
AG
GG
B
Baseline HDL Cholesterol rs693A/G
HDL Cholesterol mg/dl
80
60
40
20
0
AA
AG
GG
Week 24 HDL Cholesterol rs693A/G
HDL Cholesterol mg/dl
100
80
60
40
20
0
AA
AG
GG
C
Baseline Glucose rs693A/G
Blood Glucose mg/dl
300
200
100
0
AA
AG
GG
Week 24 Glucose rs693A/G
Blood Glucose mg/dl
300
200
100
0
AA
AG
GG
D
Baseline LDL Cholesterol rs693A/G
LDL Cholesterol mg/dl
250
200
150
100
50
0
AA
AG
GG
Week 24 LDL Cholesterol rs693A/G
LDL Cholesterol mg/dl
250
200
150
100
50
0
AA
AG
GG

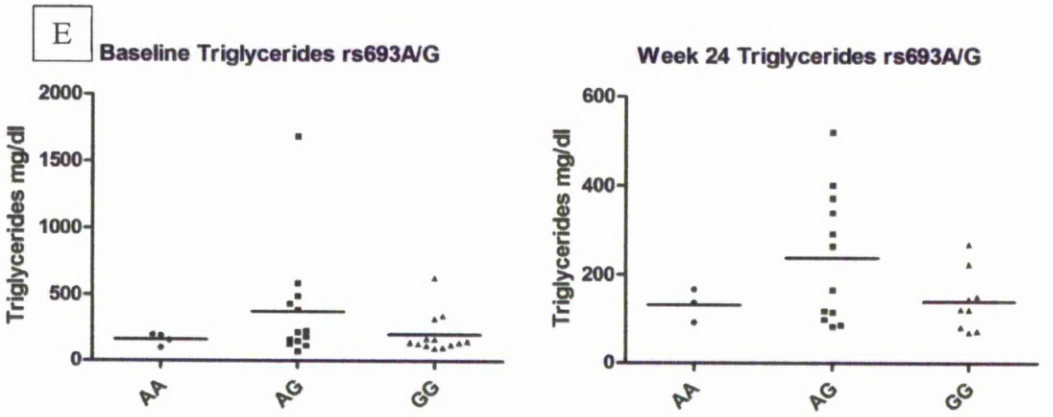


Figure 6.17: - LDLR SNP rs1529729C/T - Analysis of genotype influence on lipid parameters: A= Comparison of Baseline Total Cholesterol levels against Week 24 Total cholesterol levels (mg/dl); B= Comparison of Baseline HDL-C levels against Week 24 HDL-C levels (mg/dl); C= Comparison of Baseline Fasted Blood Glucose levels against Week 24 Fasted Blood Glucose levels (mg/dl); D= Comparison of Baseline LDL-C levels against Week 24 LDL-C levels (mg/dl); E= Comparison of Baseline TG levels against Week 24 TG levels (mg/dl) Median values denoted by black line.

The distribution of the genotypes for the rs7575840G/T SNP within ApoB included 22 individuals with the GG genotype (14 male and 8 female) and 10 heterozygotes with the GT genotype (3 male and 7 females). Figure 6.18 below shows the cycle threshold analysis of the rs7575840G/T allelic discrimination assay. Univariate analysis showed a trend between the T allele and HDL cholesterol levels at both baseline and week 24 (P=0.074 and P=0.083 respectively), caution is needed in the interpretation of this association in the absence of homozygous TT patients and the low numbers of patients in the cohort. There was a significant association with lower baseline and week 24 triglyceride parameters for homozygous wild type GG genotype (P= 0.041 and P=0.003, respectively), Table 6.10.

Table 6.10: Univariate analysis of ApoB rs7575840G/T SNP in Italian Patient Cohort on Metabolic Parameters (n=32)

	GG	GT	TT	P Value
Patient Numbers	22	10	0	
Baseline Total Cholesterol (mg/dl) Median	167	245	NA	0.625
Baseline HDL Cholesterol (mg/dl) Median	35	46	NA	0.074
Baseline Blood Glucose (mg/dl) Median	82	87	NA	0.512
Baseline LDL Cholesterol (mg/dl) Median	96	136	NA	0.311
Baseline Triglycerides (mg/dl) Median	142	178	NA	0.041
W24 Total Cholesterol (mg/dl) Median	172	210	NA	0.42
W24 HDL Cholesterol (mg/dl) Median	39	43	NA	0.083
W24 Blood Glucose (mg/dl) Median	90	87	NA	0.354
W24 LDL Cholesterol (mg/dl) Median	101	131	NA	0.438
W24 Triglycerides (mg/dl) Median	120	151	NA	0.003
Gender % (M/F)	64/36	30/70	0/0	

Figure 6.19, below, shows the distribution of baseline lipid parameters for rs7575840G/T compared to the corresponding week 24 parameters by genotype with median parameter values highlighted within each scatter plot.

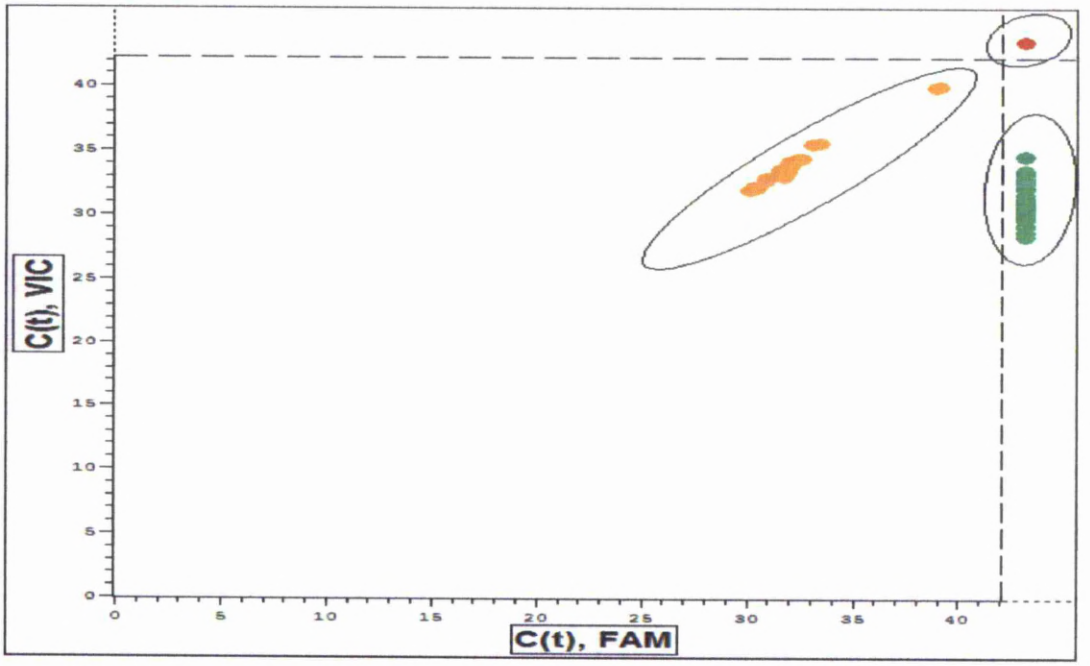
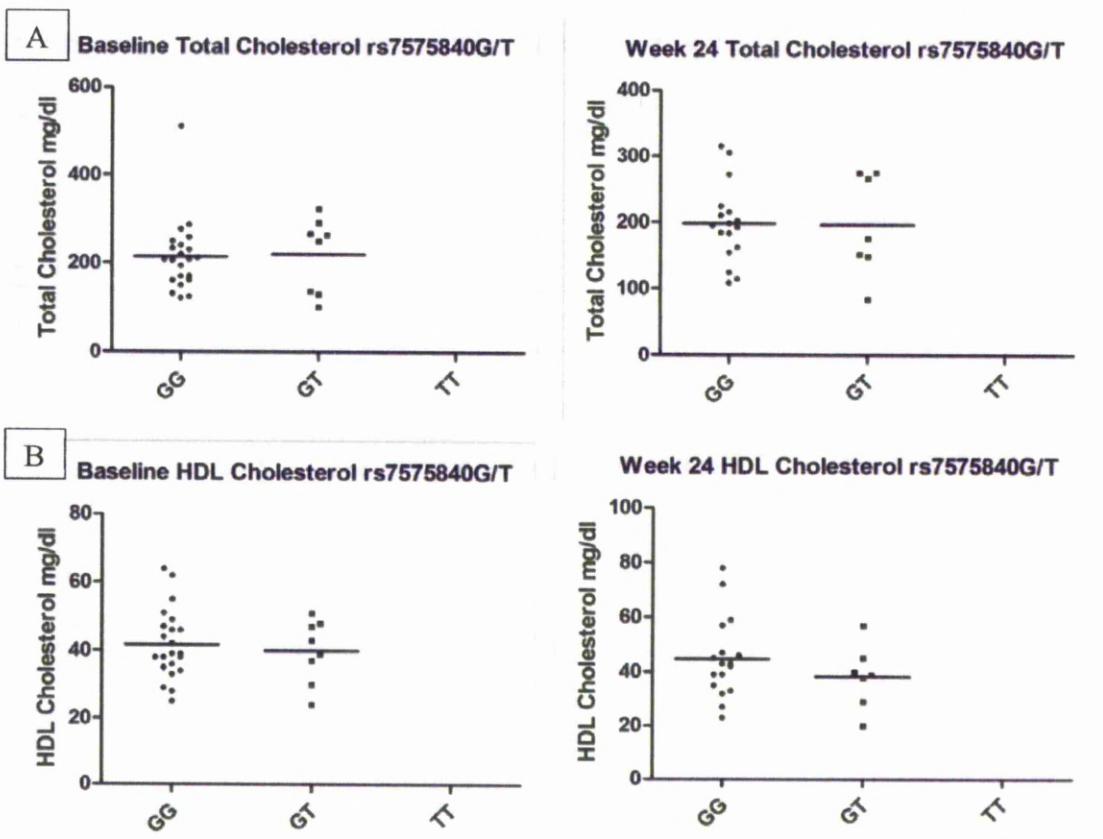


Figure 6.18: ApoB rs7575840G/T - Cycle threshold analysis of allelic discrimination assay by qPCR.



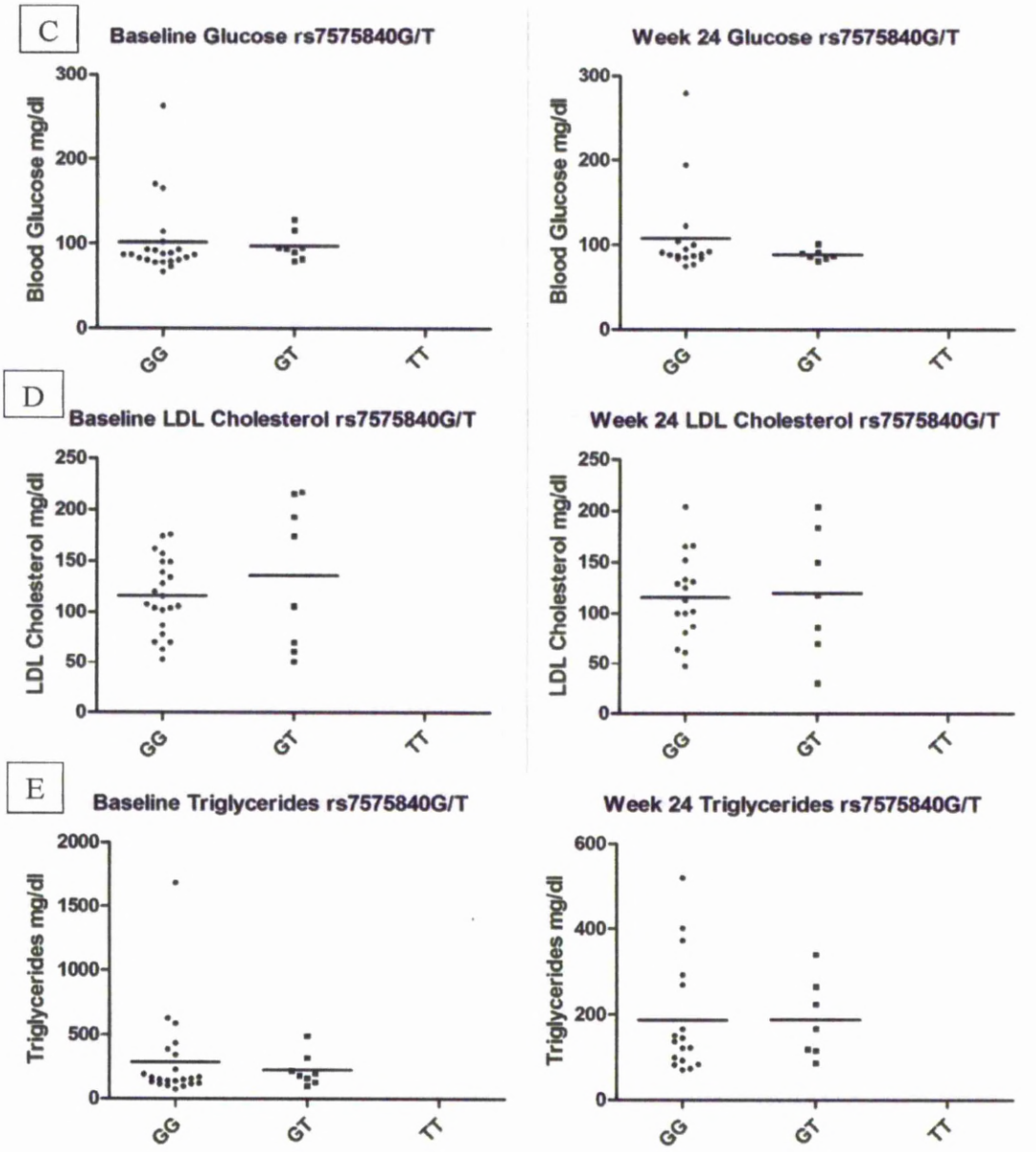


Figure 6.19: - LDLR SNP rs1529729C/T - Analysis of genotype influence on lipid parameters: A= Comparison of Baseline Total Cholesterol levels against Week 24 Total cholesterol levels (mg/dl); B= Comparison of Baseline HDL-C levels against Week 24 HDL-C levels (mg/dl); C= Comparison of Baseline Fasted Blood Glucose levels against Week 24 Fasted Blood Glucose levels (mg/dl); D= Comparison of Baseline LDL-C levels against Week 24 LDL-C levels (mg/dl); E= Comparison of Baseline TG levels against Week 24 TG levels (mg/dl) Median values denoted by black line.

6.5.9 Multivariate Analysis of SNPs and Associations with Metabolic Parameters

Table 6.11 below summarises the associations of the SNPs in the metabolic genes: ABCA1, LDLR, SCARB1, ABCG1, ABCG8 and ApoB with the key lipid parameters: total cholesterol, HDL-cholesterol, fasted blood glucose levels, LDL-cholesterol and triglycerides. P values are shown for univariate and multivariate associations.

Multivariate logistic regression confirmed significant associations with the A allele in ABCA1 rs2230806G/A SNP at week 24 for elevated total cholesterol (P=0.04) and increased LDL-C (P=0.03) Figure 6.3 (A and D) above, correlated with age (P=0.0004), height (P=0.02) and five other SNPs (see sections 6.5.4 - 6.5.8) in LDLR (rs1529729C/T, C allele, P=0.0002), SCARB1 (rs2278986C/T, T allele, P=0.0006), ABCG1 (rs1893590A/C, A allele, P=0.0072), ABCG8 (rs6544713C/T, T allele, P=0.0001) and ApoB (rs693A/G, G allele, P=0.003), Table 6.9.

Multivariate backward logistic regression analysis of the LDLR rs1529729C/T SNP confirmed a highly significant association of the TT genotype with lower baseline TG levels, (P=0.0008), an association which was maintained at week 24 TG levels (P=<0.00001), see Figure 6.7 (E). The T allele was also highly significantly associated with lower total cholesterol (P=0.0002) and lower LDL-C (P=0.0001) at week 24, see Figure 6.7 (A and D), correlated with height (P=0.02) and two other SNPs, a second in LDLR (rs2228671C/T, C allele, P=0.05), although this association should be interpreted with caution as only 2 patients were heterozygous for the T allele and there were no homozygous TT patients in the cohort (see Figure 6.8, (A

and D)); and the other in ABCG1 (rs1893590A/C, C allele, P=0.0014), see section 6.5.6, although the number of carriers of the C allele is low (7) so caution should be exercised when interpreting this association. Multivariate logistic regression analysis of the LDLR rs2228671C/T SNP showed a significant association of the T allele with lower baseline TG levels, (P=0.032), and at week 24 in TG levels (P=0.0001), see Figure 6.8 (E), Table 6.11. Although, as previously discussed, interpretation of this association should be with caution as only 2 patients carried the T allele for this SNP in the patient cohort.

Multivariate analysis of the SCARB1 rs2278986C/T SNP also confirmed highly significant associations with the T allele at week 24 for elevated total cholesterol (P=0.0006), decreased HDL-C (P=<0.00001) and elevated LDL-C (P=0.0005) Figure 6.11 (A, B and D), correlated with age (P=0.0004), height (P=0.02) and five other SNPs one in ABCA1 (rs2230806G/A, A allele), ABCG8 (rs6544713C/T, T allele) and ApoB (rs693A/G, G allele) and LDLR (rs1529729C/T, C allele), as previously discussed; and ABCG1 (rs1893590A/C, C allele, P=0.0072, see 6.5.6).

Multivariate backward logistic regression analysis of the ApoB rs693A/G SNP confirmed significant associations with the G allele at week 24 for elevated total cholesterol (P=0.0003), increased HDL-C (P=0.0029) increased LDL-C (P=0.0002), and increased TG levels (P=0.0012), Figure 6.17 (A, B, D and E), correlated with age (P=0.0004), height (P=0.0242) and five other SNPs, (previously discussed in relation to total cholesterol and LDL-cholesterol), one in ABCA1 (rs2230806G/A, A allele, P=0.04), one in LDLR (rs1529729C/T, C allele, P=0.0002), SCARB1

(rs2278986C/T, T allele, P=0.0006), ABCG1 (rs1893590A/C, C allele, P=0.0072), and ABCG8 (rs6544713C/T, T allele, P=0.0001). Multivariate logistic regression analysis of ApoB rs7575840G/T SNP confirmed significant associations with the T allele at week 24 for total elevated TG levels (P=0.0003) Figure 6.19 (E), correlated with age (P=0.0017), and five other SNPs, two in LDLR (rs1529729C/T, C allele, P=<0.00001, and rs2228671C/T, C allele, P=0.0001, see 6.5.4), ABCG1 (rs1893590A/C, C allele, P=0.0082, see 6.5.6), and ABCG8 (rs6544713C/T, T allele, P=0.004, see 6.5.7) and ApoB (rs693A/G, G allele, P=0.0012, see above).

Table 6.11: Summary analysis of metabolic SNPs in Italian Patient Cohort on Metabolic Parameters (n=32). Legend: Uni = Univariate, Multi = Multivariate, Blank box = No significance (per multivariate backward logistic regression analysis), BTC = Baseline Total Cholesterol; BHDL = Baseline HDL Cholesterol, BBG = Baseline Blood Glucose, BLDL = Baseline LDL Cholesterol, BTGs = Baseline Triglycerides, W24TC = Week 24 Total Cholesterol; W24HDL = Week 24 HDL Cholesterol, W24BG = Week 24 Blood Glucose, W24 LDL = Week 24 LDL Cholesterol, and W24TGs = Week 24 Triglycerides. Statistical significance is shown where P =<0.05 and a Trend is where P = ≤0.1

	ABCA1 rs2230806GA		**LDLR rs1529729CT**		**LDLR rs2228671CT**		**SCARB1 rs2278986CT**		**ABCG1 rs1893590AC**		**ABCG8 rs6544713CT**		**ApoB rs693AG**		**ApoB rs7575840GT**	
	P Value		**P Value**		**P Value**		**P Value**		**P Value**		**P Value**		**P Value**		**P Value**	
	Uni	**Multi**	**Uni**	**Multi**	**Uni**	**Multi**	**Uni**	**Multi**	**Uni**	**Multi**	**Uni**	**Multi**	**Uni**	**Multi**	**Uni**	**Multi**
BTC	0.472	0.0419	0.415	0.004	0.187	0.011	0.155	0.003	0.472	0.029	0.538		0.995		0.625	
BHDL	0.835		0.556		0.139	0.008	0.529	0.00001	0.835		0.095	0.005	0.493		0.074	0.002
BBG	0.488	0.038	0.607	0.018	0.379	0.02	0.695	0.011	0.488	0.038	0.508	0.097	0.92		0.512	0.076
BLDL	0.581		0.993		0.193	0.001	0.867		0.581		0.472		0.389	0.022	0.311	0.016
BTGs	0.161		0.22	0.0008	0.059	0.032	0.183		0.161	0.013	0.056	0.005	0.07	0.001	0.041	
W24TC	0.961	0.04	0.6	0.0002	0.813		0.597	0.0006	0.7	0.0072	0.892	0.0001	0.476	0.0003	0.42	
W24HDL	0.788		0.394		0.928		0.098	<0.00001	0.87		0.441		0.777	0.0029	0.083	
W24BG	0.854	0.0419	0.615		0.993		0.772		0.702		0.095	0.054	0.823		0.354	
W24LDL	0.821	0.03	0.941	0.0001	0.971		0.53	0.0005	0.531	0.0014	0.751	0.0001	0.602	0.0002	0.438	
W24TGs	0.514	0.0301	0.011	<0.0001	0.484	0.0001	0.336		0.453	0.0082	0.246	0.004	0.215	0.0012	0.003	0.0003

6.6 Discussion

This chapter sought to validate the importance of eight known polymorphisms in six metabolic genes which had previously been associated with lipid abnormalities and cardiovascular risk in a HIV+ cohort who had been switched to a lipid sparing regimen of un-boosted ATV due to prior display of HADL characteristics. Although repeat measures were taken during clinic visits for a period up to 96 weeks, patient attendance for clinic visits was poor and the findings should also be interpreted in the context of this and the small numbers involved.

The rs2230806G/A SNP in ABCA1 had previously been shown to be associated with elevated LDL-C levels in HIV- men who were homozygous AA (Abellan *et al.*). This study only had four participants with that genotype, three of which were female. It was interesting therefore to find that elevated LDL-C in the HIV+ cohort was more prevalent in females with at least one copy of the A allele, than we had expected, with values in the range 102mg/dl - 216mg/dl with 9/12 in the range borderline high to very high as defined in section 6.1.2 above. An association with elevated LDL-C in men with at least one copy of the A allele was more established in individuals who carry at least one copy of the A allele in the ABCA1 SNP rs2230806G/A and are also homozygous GG genotype for the rs7575840GT SNP in ApoB 8/9 patients with LDL-C in the range 70mg/dl - 176mg/dl. Eight of the same female population were also homozygous GG genotype for the rs7575840GT SNP in ApoB and all had elevated LDL-C in the range borderline high to very high (134mg/dl – 193mg/dl). We hypothesise therefore that the combination of the GG genotype for ApoB rs7575840GT and a single copy of the A allele from the ABCA1 rs2230806G/A SNP

is sufficient to result in elevated LDL-C in Caucasian HIV+ patients. This confirms the association with increased risk previously reported in individuals with a combination of these two SNPs (Kathiresan *et al.*, 2008). Further investigations in a larger population would need to be undertaken to confirm the hypothesis that this association is gender independent in a HIV+ cohort. Such confirmation may provide a future predictor of HADL risk in HIV+ individuals and provide insights into lipid-sparing alternative therapies.

We were also able to confirm associations seen in HIV negative populations with other SNP combinations and other lipid parameters. It has previously been reported that the two SNPs in LDLR, (rs2228671C/T and rs1529729C/T) were associated with elevated LDL-C levels and an increased risk of developing coronary artery disease (Kathiresan *et al.*, 2008; Linsel-Nitschke *et al.*, 2008). The former has also been associated with hypercholesterolaemia (Aulchenko *et al.*, 2009). We observed an association between the C allele in the LDLR rs1529729C/T SNP and elevated total cholesterol, increased LDL-C and higher TG levels. When this SNP was combined with the GG genotype for ApoB rs7575840GT, 11/12 patients had elevated total cholesterol in the range 206mg/dl – 511mg/dl (borderline – high as defined in Section 6.1.2 above), 9/12 patients in the same group also had high LDL-C in the range 130mg/dl - 176mg/dl. Baseline TG levels were in the range 166mg/dl – 1685mg/dl borderline high to very high (per Section 6.1.2 above) in the same patient group. In the same group 11/12 patients were also homozygous CC for the LDLR SNP rs2228671C/T with the twelfth patient heterozygous CT at the same SNP. This confirms, therefore, the increased risk associated with a combination of these three SNPs (Kathiresan *et al.*, 2008). Again further investigations in a larger

population would be required to confirm the hypothesis that these associations are prevalent in a larger HIV+ cohort. Such confirmation may prove a useful tool for clinicians requiring a predictor of HADL risk in HIV+ individuals.

Our investigations were unable to confirm the inverse association with HDL-C levels previously reported as a consequence of reduced SRB1 protein levels linked with the C allele in the SCARB1 rs2278986C/T SNP (West *et al.*, 2009). The cohort contained 2 homozygous CC genotypes (both female), and 22 heterozygotes carrying one C allele but HDL-C levels were in the range 24mg/dl to 55mg/dl for the 17 men in the cohort with only 1/17 exhibiting high HDL-C levels (>50mg/dl), and in the range 25mg/dl – 64mg/dl for the 11 women with at least one copy of the C allele with only 2/11 exhibiting high HDL-C levels (>60mg/dl). We were unable to confirm an association with this SNP in the homozygous TT patients which may have indicated an opposite effect in HIV+ populations. We were able to confirm an association with decreased HDL-C in patients with the T allele when it was also associated with a combination of other SNPs and demographic factors including age, height and the following SNPs: ABCA1 rs2230806G/A, A allele; LDLR rs1529729C/T, C allele; ABCG1 rs1893590A/C, C allele; ABCG8 rs6544713C/T, T allele; and ApoB rs693A/G, G allele. The same combination was also confirmed as associated with elevated total cholesterol and increased LDL-C after 24 weeks on a lipid-sparing regimen of un-boosted ATV in 24/32 patients in the HIV+ cohort. Whilst previous studies have reported an increased risk of combined SNPs in non-HIV related metabolic disorders, it is necessary to remain cautious when predicting the same outcome in larger HIV+ cohorts without further validation of these findings. If further validation confirms these findings in larger populations and

different ethnicities, these SNPs could form the basis of a matrix for predicting HADL outcomes.

We were also unable to confirm the association between the AA genotype of the rs1893590A/C SNP within ABCG1 with lower HDL-C levels in women as previously reported in non-HIV patients (Abellan *et al.*). We only observed 9 females in the cohort with the AA genotype and their HDL-C levels were in the range 39mg/dl – 62mg/dl compared to a range of 25mg/dl – 64mg/dl in all females and the combined genders group. The lack of association may be attributed to the small numbers, but the trend in the group was towards higher levels of HDL-C in the homozygous AA females than for the heterozygous females and for all genotypes in the male group. An association was shown between reduced baseline TG values and the C allele although again the numbers were low (7) and validation in a larger cohort is required. Elevated total cholesterol and increased LDL-C levels were associated with the C allele at week 24 when the SNP was grouped with a number of demographic factors and other SNPs including: age; height; and a combination of five SNPs: ABCA1 (rs2230806G/A, A allele); LDLR (rs1529729C/T, C allele); SCARB1 (rs2278986C/T, T allele); ABCG8 (rs6544713C/T, T allele); and ApoB (rs693A/G, G allele).

The rs693A/G SNP within ApoB has previously been associated with increased LDL-C levels and increased risk of cardiovascular events, especially when combined with SNPs at rs1529729C/T, (C allele) in LDLR and rs7575840G/T (T allele) (Kathiresan *et al.*, 2008). We were able to confirm this finding in the HIV+ cohort associated with the G allele in rs693SA/G. At week 24 we determined an association

of the G allele with elevated total cholesterol, increased HDL-C levels and LDL-C levels and elevated TG levels. These correlations were statistically significant when the SNP was correlated with demographic factors age and height together with five other SNPs: ABCA1 (rs2230806G/A, A allele); LDLR (rs1529729C/T, C allele); SCARB1 (rs2278986C/T, T allele); ABCG1 (rs1893590A/C, C allele); and ABCG8 (rs6544713C/T, T allele).

In summary, this chapter describes the association of known polymorphisms in metabolic genes which have previously been linked with lipid abnormalities in non-HIV populations. The role that these polymorphisms have in influencing characteristics of HADL in five key lipid parameters: total cholesterol, HDL-C, Blood glucose (fasted), LDL-C and TGs in a Caucasian HIV+ cohort and the potential influence that these findings may have as future predictors of HADL risk now requires further validation in larger cohorts.

CHAPTER 7

GENERAL DISCUSSION

7.1 General Discussion

Despite the advances in ARVs to treat HIV over the last 20 years, there is still no cure or vaccine. HIV+ individuals have a lifelong treatment burden to suppress their viral loads to below detectable levels. Long term treatment with HAART has greatly improved patient mortality and morbidity outcomes (Falutz, 2007), but long-term HAART is widely associated with cardiovascular and metabolic complications including HADL (Behrens *et al.*, 2000; Bonfanti *et al.*, 2000; Carr, 2000; Mallon, 2007; Mallon *et al.*, 2003). PIs, NRTIs and NNRTIs, have all been implicated in HADL, although PIs are the most widely associated (Behrens *et al.*, 2000; Carr, 2000; Carr *et al.*, 1998; Falutz, 2007; Mallon, 2006). Insulin resistance is common with PIs and NRTIs (Falutz, 2007; Lagathu *et al.*, 2005) and they have previously been shown to impact several key lipid metabolism molecular pathways including intra-nuclear transcription factors and the nuclear proteasome (Lagathu *et al.*, 2005; Mallon, 2007).

The primary focus of this PhD thesis was to determine the direct effects of the PIs RTV and ATV on the metabolic NRs involved in lipid metabolism, by *in vitro* methods, with a specific focus on the roles of FXR, LXRα and PPARα in the metabolic complications associated with the use of these drugs. The secondary focus of this thesis was to establish the importance of known polymorphisms in the LXRα gene and eight known polymorphisms in six metabolic genes (ABCA1, LDLR, SCARB1, ABCG1, ABCG8 and ApoB) which had previously been associated with lipid abnormalities and cardiovascular risk in non-HIV+ populations (Abellan *et al.*; Aulchenko *et al.*, 2009; Dahlman *et al.*, 2009; Kathiresan *et al.*, 2008; Legry *et al.*,

2008; Linsel-Nitschke *et al.*, 2008; Price *et al.*, 2011; West *et al.*, 2009) in HIV+ cohorts with confirmed HADL complications. The patients in the Italian HIV+ cohort were switched to a lipid-sparing regimen of un-boosted ATV at baseline, due to prior display of HADL characteristics. This cohort was probed to validate the importance of the eight polymorphisms in ABCA1, LDLR, SCARB1, ABCG1, ABCG8 and ApoB.

High levels of toxicity associated with RTV mean that it is no longer used at therapeutic doses. This has been counterbalanced by the availability of other PIs with more attractive lipid profiles (Foisy *et al.*, 2008) including ATV (Barreiro *et al.*, 2005). RTV, as a pharmacoenhancer, was reported as a notorious mediator of drug-drug interactions and toxological effects due to non-clearance of these drugs (Barreiro *et al.*, 2005; Foisy *et al.*, 2008). Patients taking a RTV boosted regimen incurred significant increases in total cholesterol and triglyceride levels following 48 week trials, whereas the same parameters in the ATV cohort were unchanged (Barreiro *et al.*, 2005). In Chapter 2, initial studies determined that the cellular accumulation of ATV was 2 fold greater than that of RTV in the Huh7 cell line. This was an important discovery because it determined that the metabolic disruption and the deleterious side effects associated with RTV, compared to the more favourable lipid profile of ATV, were not due to higher intracellular accumulation of the former.

Gene expression studies, in Chapter 2, determined that LXRα was significantly up-regulated, in the presence of RTV, and it was a master regulator of key downstream target gene expression, including SREBP-1c, which was previously implicated in PI induced lipodystrophy (Telenti *et al.*, 2002). The role of LXRα in the regulation of

target gene expression was under-pinned by co-incubation of RTV with the LXRα antagonist 22(S)-hydroxycholesterol. In Chapter 3, the effects of RTV on LXRα target gene expression, observed in Chapter 2, were validated by the use of siRNA to knockdown LXRα gene expression. LXRα knockdown was also confirmed at the protein level in Chapter 3.

In Chapter 2, FXR gene expression was also significantly up-regulated in the presence of RTV. The effects on downstream target genes of FXR were consistent with its up-regulation, with significant up-regulation of ApoC-II and significant down-regulation of HNF4α gene expression observed. Co-incubation with the LXRα antagonist 22(S)-hydroxycholesterol did not inhibit the up-regulation of FXR or the down-regulation of HNF4α by RTV. The up-regulation of ApoC-II by RTV was, however, completely abolished in the presence of the LXRα antagonist which indicated more than one mechanism was involved in its up-regulation. The observed significant down-regulation of ApoC-II, a mediator of TG metabolism, by ATV may be attributed to regulation of this gene by PPARα. This may present a future opportunity for further investigation, but was not further probed in these studies as the focus was on the deleterious metabolic side effects associated with RTV. In Chapter 3, incubation of RTV in the biological knockdown of LXRα by siRNA samples did not prevent the up-regulation of FXR or the down-regulation of HNF4α. The up-regulation of ApoC-II by RTV was, however, completely abolished in the siRNA knockdown of LXRα samples which supported the hypothesis that more than one mechanism was involved in its up-regulation in response to RTV.

Initial gene expression studies in Chapter 2 also demonstrated that incubation with ATV resulted in significant down-regulation of FXR, whilst HNF4α gene expression was significantly up-regulated by ATV suggesting that down-regulation of FXR may have a role in this response. This was the opposite effect to that observed for RTV at these genes. This was an important discovery because it demonstrated that the gene expression response to RTV and ATV of two key metabolic nuclear receptors, FXR and HNF4α, had contrasting profiles, which may provide an insight into the contrasting lipid profiles of the two PIs.

FXR has been reported as a down-regulator of SREBP-1c gene expression (via SHP-1), a mechanism reported to have positive effects on glucose metabolism (Fiorucci *et al.*, 2007). As FXR was up-regulated by RTV, a down-regulation of SREBP-1c gene expression was anticipated, if FXR was the master regulator of its gene expression. The investigations in Chapter 2 resulted in the opposite effect, however, with RTV significantly up-regulating SREBP-1c. This indicated that another mechanism was involved with SREBP-1c up-regulation. We hypothesised that this mechanism was the observed significant up-regulation of LXRα by RTV. When the hypothesis was tested by the inhibition of LXRα by the antagonist 22(S)-hydroxycholesterol (Chapter 2) and biological knockdown by siRNA (Chapter 3) the effects of RTV on both LXRα and SREBP-1c were abolished supporting the hypothesis. This is important because the up-regulation of SREBP-1c was previously reported to be involved in PI induced lipodystrophy (Bastard *et al.*, 2002; Telenti *et al.*, 2002) and its up-regulation leads to increased production of triglycerides and lipogenesis. Elevated triglyceride levels are key clinical concern in HADL and other metabolic

disorders. Furthermore SREBP-1c gene expression was significantly down-regulated at all concentrations of ATV suggesting a potential explanation for the contrasting lipid profiles of ATV and RTV.

Interestingly, in Chapter 3, biological knockdown of LXRα by siRNA resulted in down-regulation of LXRα gene expression in the presence of all RTV. This is important because it could provide a potential future therapeutic mechanism for inhibition of LXRα *in vivo,* either by chemical antagonism of therapeutic use of siRNA. This would be a viable option if the issues which were previously associated with increased production of liver TGs and increased lipogenesis by LXRα agonists (Bradley *et al.*, 2005; Calkin *et al.*) can be avoided. As interest develops in the use of siRNA for gene therapy for complex metabolic disorders these biological mediators of inhibition will provide opportunities to specifically regulate key therapeutic targets without the "off-target" side effects widely associated with drugs in this class (Lee *et al.*, 2003; Rader, 2007).

LXRα was reported to inhibit PPARα signalling (Miyata *et al.*, 1996). It was hypothesised that LXRα was regulating PPARα in the RTV gene expression studies (Chapter 2). PPARα gene expression was significantly down-regulated by RTV, however, inhibition of LXRα by 22(S)-hydroxycholesterol and by biological knockdown by LXRα siRNA abolished these effects on PPARα. This was important because it supported the hypothesis that LXRα is a key player in the regulation of PPARα. The down-regulation of PPARα by ATV observed at 10μM (maximal decrease of 1.1 fold compared to vehicle) was to a lesser magnitude than observed in the RTV experiments. It was proposed that this reflected the magnitude of difference

between the observed ATV-mediated increase in LXRα expression and the observed RTV-mediated increase in LXRα expression.

It was reported that PPARα induction leads to the repression of ApoC-III (Duval *et al.*, 2007; Feldman *et al.*, 2008; Yamada *et al.*, 2007). Down-regulation of PPARα was observed in the RTV assays. It was hypothesised that this contributed to the up-regulation of ApoC-III, as LXRα up-regulated ApoC-III through its down-regulation of PPARα. This hypothesis was confirmed when the inhibition of LXRα by 22(S)-hydroxycholesterol and biological knockdown by LXRα siRNA abolished the effects of RTV on ApoC-III (and PPARα) supporting the role of LXRα as a key player in the regulation of both ApoC-III and PPARα. This was important because ApoC-III and PPARα are involved with TG metabolism (Cariou *et al.*, 2007; Francis *et al.*, 2003) and ApoC-III was implicated in elevated TG levels in HIV+ men (Mallon, 2007), furthermore ApoC-III forms a major constituent of very low density lipoprotein (VLDL), levels of which have been elevated in HIV+ cohorts and healthy volunteer cohorts with short term exposure to RTV (Mallon, 2007).

It was previously reported that ApoC-III was involved with the modulation of ApoA-I in transgenic mice (Kan *et al.*, 2004), and that PPARα was also involved with its regulation (Berger *et al.*, 2002; Francis *et al.*, 2003; Lee *et al.*, 2003). PPARα has been implicated in the up-regulation of ApoA-I (Tobin *et al.*, 2006). ApoA-I was significantly up-regulated by RTV, which led us to probe whether LXRα was directly or indirectly involved with its observed regulation. It was hypothesised that up-regulation of LXRα or a combination of its target genes could be the mechanism involved with ApoA-I up-regulation. Use of the LXRα antagonist, 22(S)-

hydroxycholesterol and in the biological knockdown of LXRα by siRNA abolished the up-regulation of ApoA-I, supporting this hypothesis. Regulation of ApoA-I gene expression is important because it encodes apolipoprotein A-I, which is the major protein component of HDL and a key component of reverse cholesterol transport. LXRα, PPARα, FXR and VDR are all regulators of ApoA-I gene expression. These studies have demonstrated that LXRα is a master regulator in the presence of RTV *in vitro* but further studies are required to determine the implications of inhibition of LXRα *in vivo* on its up-regulation of ApoA-I and its metabolic outcome.

LXRα also represses VLDLR (Scotti *et al.*) which was significantly down-regulated by RTV. Inhibition of LXRα with 22(S)-hydroxycholesterol abolished the effects of RTV on VLDLR which supported the hypothesis that LXRα was regulating VLDLR expression. Biological knockdown of LXRα by siRNA underpinned this hypothesis as the impact of RTV on VLDLR gene expression was diminished, in Chapter 3. The up-regulation of VLDLR by ATV was consistent with the regulation by FXR and by PPARα. This may again underpin the conclusion that these different mechanisms are involved in the contrasting lipid profiles of ATV and RTV.

Chapter 2 and Chapter 3 of this thesis demonstrated the involvement of LXRα in the differing target gene expression profiles associated with the PIs RTV and ATV. These chapters also demonstrated that inhibition of LXRα by a chemical antagonist and by biological knockdown using siRNA can be used to confirm the target genes for which LXRα is a regulator, with specific focus on the responses to RTV and ATV. The endogenous role of LXRα is as a master regulator of cholesterol homeostasis in the liver and macrophages (Gronemeyer *et al.*, 2004a; Kalaany *et al.*,

2006; Pascussi *et al.*, 2008). It facilitates the processing and storage of cholesterol and has been implicated in the increased expression of the ATP binding cassette proteins ABCA1, ABCG1, ABCG5 and ABCG8, key players in cholesterol transport and metabolism (Eloranta *et al.*, 2005; Pascussi *et al.*, 2008; Tirona *et al.*, 2005). These studies have demonstrated that LXRα is a master regulator in the presence of RTV *in vitro* but further studies are required to contrast the effects of RTV on LXRα with those of an endogenous ligand such as 24(S)-hydroxycholesterol and the synthetic LXR agonists T0901317 and GW3965 (Tocris Biosciences), although as all are active at both the LXRα and LXRβ isoforms, further analysis will be required to ensure the observed findings are due to agonist effects on LXRα. Confirmation could be obtained through the use of siRNA knockdown of LXRβ. Further analysis should also be undertaken *in vivo* to confirm the *in vitro* results in a LXRα knockout humanised murine model. The use of specific targeting siRNA knockdown of hepatic LXRα would have greater potential as a therapeutic measure than chemical antagonists, albeit the method of delivery would require careful design. Chemical antagonists would be more likely to have unwanted systemic effects targeting LXRs throughout the body resulting in inhibition of the pathways key to reverse cholesterol transport and affecting the formation nascent HDL cholesterol particles.

Chapter 4 of this thesis focused on the key hepatic transporters involved in cholesterol homeostasis, bile acid metabolism and in glucose transport, some of which also play key metabolic roles in the periphery and the intestine. This chapter investigated the relative impact that LXRα regulation has on cholesterol and bile acid

transporters to determine its potential contribution to the deleterious metabolic complications associated with PI based HAART regimens.

ABCA1 is a mediator of cholesterol and phospholipid efflux to lipid poor apolipoproteins for the formation of nascent HDL-cholesterol (HDL-c) (Borst *et al.*, 2002). ABCA1 is a direct target of LXRα (Edwards *et al.*, 2002). It was predicted that ABCA1 gene expression would be up-regulated by RTV and was hypothesised that this up-regulation could be inhibited by antagonism of LXRα. This hypothesis was confirmed in the RTV plus 22-(S)-hydroxycholesterol assays, and validated by the biological knockdown of LXRα by siRNA. Interestingly, the down-regulation of ABCA1 gene expression in the RTV plus 22-(S)-hydroxycholesterol assays provided an insight into other mechanisms regulating the gene and showed LXRα to be a primary regulator of ABCA1 gene expression. It was hypothesised that the co-repressor molecules NCoR and SMRT may be implicated in the down-regulation of ABCA1 expression in the presence of the LXRα antagonist, as a regulatory role for these co-repressors has already been reported (Kalaany *et al.*, 2006). Further investigation would be required to underpin this hypothesis, but this was outside the scope of this thesis.

ABCB1 was previously shown to be involved in the transport of both RTV and ATV (Perloff *et al.*, 2005; Solon *et al.*, 2002). Down-regulation of ABCB1 gene expression by RTV and the increased gene expression in the presence of LXRα antagonism and knockdown suggested LXRα may be involved in ABCB1 gene regulation. It was postulated that LXRα may compete with other nuclear receptors, which may include PXR and HNF4α (Pascussi *et al.*, 2008; Tirona *et al.*, 2005), at

the ABCB1 binding site. This may partially explain the differential gene expression response in the presence of known PXR agonists (RTV and ATV), and LXRα agonists (RTV), and the LXRα antagonist (22-(S)-hydroxycholesterol), although further investigations which were outside the scope of this study would be required to underpin this theory. The results from Chapter 4 suggested that there may be some merit in investigating the effects of PXR knockdown on the ABCB1 transporter response and to look at the effects of dual receptor knockdown for both PXR and LXRα on the gene expression of ABCB1 in the presence of RTV. This interesting further investigation, however, was outside the scope of this thesis.

The up-regulation of ABCB2, a key player in bile acid transport, suggested a role for regulation by FXR, however, at higher RTV concentrations down-regulation of ABCB2 gene expression was observed which implicated a role for regulation by LXRα. The effects of the up-regulation at 0.1μM RTV were blocked by co-incubation with the LXRα antagonist and by biological knockdown of LXRα. At higher RTV concentrations biological knockdown and chemical antagonism of LXRα resulted in only small differences in the regulation of ABCB2 supporting the hypothesis that although LXRα played a role in the regulation of this transporter it was not the master regulator of this gene.

ABCB11, the bile salt efflux pump, was previously shown to be regulated by FXR (Edwards *et al.*, 2002; Kalaany *et al.*, 2006). Significant up-regulation of ABCB11 gene expression was observed at 0.1μM RTV, which may be consistent with the up-regulation of FXR by RTV and its previously reported role in ABCB11 regulation (Edwards *et al.*, 2002; Kalaany *et al.*, 2006). Further probing of the role of FXR

would be required to confirm this association but this was outside the scope of these investigations. At higher RTV concentrations, however, down regulation of ABCB11 gene expression was evident, furthermore the LXRα antagonist, 20μM 22-(S)-hydroxycholesterol, reversed the effects of RTV on ABCB11. This supported the hypothesis that LXRα played a key role in the regulation of ABCB11. This hypothesis was also validated by the results of the siRNA LXRα knockdown assays where the effects of RTV on ABCB11 were diminished.

Down-regulation of ABCC1 gene expression by RTV was observed. Inhibition of LXRα by the antagonist 22-(S)-hydroxycholesterol and siRNA knockdown abolished or diminished this down-regulation in a concentration dependent manner. ABCC1 gene expression was also significantly down-regulated in the presence of all concentrations of ATV, suggesting a complex array of mechanisms involved in ABCC1 regulation. This was important because the outcome of ABCC1 regulation by both RTV and ATV was similar so it is unlikely that its regulation contributes to the contrasting profiles of these two drugs. There were still questions to be answered in respect of the complex mechanisms involved in its regulation and their impact cholesterol efflux and homeostasis, which were outside the scope of this thesis.

Up-regulation of ABCC2 gene expression was observed at lower RTV concentrations, however, at 10μM RTV significant ABCC2 down-regulation was observed. These effects were diminished by the LXRα antagonist, 22-(S)-hydroxycholesterol, and in the biological knockdown assays. This was interesting because it suggested a role for LXRα as a key regulator of the xenobiotic responses of this gene, albeit that LXRα was not the primary regulator of ABCC2. Other NRs

including PXR and FXR have been reported to be involved in ABCC2 regulation, (Ho *et al.*, 2005; Wang *et al.*, 2008).

ABCG1, a key player in cholesterol transport, was up-regulated by RTV. This was diminished in the presence of the LXRα antagonist 22-(S)-hydroxycholesterol, and in the biological knockdown assays supporting a role for LXRα in the regulation of ABCG1 gene expression. Another key player in cholesterol transport, ABCG4, was also up-regulated by RTV, effects which could be diminished in the presence of 22-(S)-hydroxycholesterol and by biological knockdown of LXRα. The regulation by LXRα of these genes could provide an insight into some of the metabolic complications associated with HADL, as genes on the pathways for both bile acid transport and cholesterol transport have been up-regulated simultaneously leading to disruption of bile acid and cholesterol homeostasis. These *in vitro* findings would need to be probed *in vivo* to validate this hypothesis.

Significant up-regulation of ABCG5 and ABCG8 gene expression was also observed in the RTV assays. The effects of which could be abolished or diminished by 22-(S)-hydroxycholesterol or siRNA knockdown of LXRα. Both of these transporters were previously cited as under the regulatory control of LXRα (Francis *et al.*, 2003). The studies in Chapter 4 confirmed that the gene expression of these metabolic transporters differs in response to RTV and ATV and that the actions of RTV can be blocked through modulation of LXRα gene expression. The results also provide further insight into the complex mechanisms by which LXRα regulates its targets in response to a RTV stimulus. SNPs in transporters and genes involved in lipid

homeostasis play a key role in the varied metabolic side-effect profiles seen in HIV+ individuals on HAART (Mallon, 2006; Telenti *et al.*, 2008).

The findings of these gene expression studies in metabolic transporters may provide valuable clues for future drug development, as agonists of LXRα could result in the disruption of a cascade of metabolic genes and downstream targets. The observed down-regulation of LXRα due to biological knockdown by siRNA has already been discussed in this chapter together with the potential for a future therapeutic mechanism for antagonism of LXRα *in vivo.*

Up-regulation of GLUT4 gene expression by RTV was observed. In the presence of 22-(S)-hydroxycholesterol, this up-regulation was diminished at 0.1μM and 10μM RTV, and abolished at 1.0μM. The biological knockdown of LXRα showed a role for LXRα in the regulation GLUT4 gene expression. Although caution should be exercised when interpreting the GLUT 4 results as the overall basal and experimental expression levels of the gene were quite low in the Huh7 cells.

Chapter 5 of this thesis discussed the importance of a known SNP in the LXRα gene, rs2279238C/T, which was previously associated with lipid abnormalities and cardiovascular risk in HIV negative (HIV-) populations (Dahlman *et al.*, 2009; Legry *et al.*, 2008; Price *et al.*, 2011) in a HIV+ cohort with confirmed HADL complications. There were no significant differences between the CC, CT and TT genotypes of the rs2279238C/T SNP in either the HADL+ or HADL- cohorts. It should be noted that the TT genotype was rare in the patient cohort with no examples in the HADL- patient group. Subset analysis of the allele frequency in the HADL+

and HADL- cohorts revealed a trend associated with the T allele and HADL symptoms. A further significant association was observed when the HADL- cohort was compared to the healthy volunteer cohort which suggested a potentially protective role for the CC genotype in this cohort, the power of this association increased when the analysis was performed on the allele frequency in the same cohorts. This suggested that the absence of the polymorphism in the HADL- cohort may confer protection against HADL symptoms. Further investigations in a larger population are needed to establish whether the association with the TT genotype previously observed in HIV- populations (Dahlman *et al.*, 2009; Legry *et al.*, 2008; Price *et al.*, 2011) influences HADL outcomes in non-Caucasian populations or is gender specific in a HIV+ cohort, as the investigated patient group was wholly Caucasian and predominantly male.

It was hypothesised that the LXRα SNP rs2279238C/T potentially influenced the risk of HADL development in Caucasian HIV+ populations and that it may be linked to increased risk of a fatal cardiovascular event where HADL complications were present. As no survival outcome or follow-up data for this cohort were available the hypothesised cardiovascular risk was unconfirmed. In order to confirm these hypotheses, that the observed SNP is associated with HADL outcomes and increased cardiovascular risk, the investigations would need to be repeated in a larger HIV+ cohort. HADL is a multifactorial disease and in this chapter a single polymorphism in a single gene was investigated. It was difficult to conclude that the presence of a single SNP in LXRα could predict future cardiovascular events in a HIV+ or HADL+ population.

Weak associations between baseline lipid parameters; total cholesterol ≥240mg/dl; triglycerides ≥200mg/dl; and blood glucose ≥100mg/dl were observed in the HADL+ cohort, although these associations were not linked to a specific genotype. Power for these associations increased with patient age and HADL status, however, these were not genotype driven. In order to validate these associations further investigations in a larger population are needed to investigate whether correlation between these parameters and a specific genotype can be established.

Knowledge of specific SNPs which increase the risk of developing metabolic disorders, including diabetes mellitus and cardiovascular disease, provides an insight into future treatment regimens so that individuals with the greatest risk of metabolic complications receive treatment which incorporates lipid-sparing ARVs. This knowledge will also influence the future development of drugs to treat HIV as more is known about the complex mechanisms which underpin HADL and individual susceptibility to its development.

Chapter 6 of this thesis determined the importance of eight known polymorphisms in six metabolic genes which were previously associated with lipid abnormalities and cardiovascular risk in a HIV+ cohort who were switched to a lipid-sparing regimen of un-boosted ATV due to prior display of HADL characteristics.

The ABCA1 rs2230806G/A SNP was previously associated with elevated LDL-c levels in men who were homozygous AA (Abellan *et al.*). This study had insufficient male participants with that genotype, so this association was unconfirmed. It was interesting, however, that elevated LDL-c in the HIV+ cohort was more prevalent in

females with at least one copy of the A allele, than had been expected. An association with elevated LDL-c in men was established in individuals who carry at least one copy of the A allele in the ABCA1 rs2230806G/A SNP and are homozygous GG genotype for the rs7575840GT SNP in ApoB. It was also interesting to observe that eight of the same female population were also homozygous GG genotype for the rs7575840GT SNP in ApoB and all had elevated LDL-c levels. It was hypothesised that the combination of the GG genotype for ApoB rs7575840GT and a single copy of the A allele from the ABCA1 rs2230806G/A SNP was sufficient to result in elevated LDL-c in Caucasian HIV+ populations; although caution is required when reporting this association due to the small patient cohort involved. The studies, however, confirmed the association with increased risk previously reported in individuals with a combination of these two SNPs (Kathiresan *et al.*, 2008). Further investigations in a larger population are needed to confirm the hypothesis that this association is gender independent in a HIV+ cohort. Such confirmation could provide a future predictor of HADL risk in HIV+ individuals and provide insights into lipid-sparing alternative therapies.

These studies also confirmed associations seen in HIV- populations with other SNP combinations and other lipid parameters. It was previously reported that the two SNPs investigated in LDLR, rs2228671C/T and rs1529729C/T were associated with elevated LDL-c levels and an increased risk of developing coronary artery disease (Kathiresan *et al.*, 2008; Linsel-Nitschke *et al.*, 2008), the former has also been associated with hypercholesterolaemia (Aulchenko *et al.*, 2009). An association with the C allele in the LDLR rs1529729C/T SNP and elevated total cholesterol, increased

LDL-c and higher TG levels was observed in these investigations. When this SNP was combined with the GG genotype for ApoB rs7575840GT, patients had elevated total cholesterol and increased LDL-c levels. In the same group 11/12 patients were also homozygous CC for the LDLR SNP rs2228671C/T with the twelfth heterozygous CT at the same SNP. This suggested that the outcomes were linked to the C allele at this SNP. The increased risk associated with a combination of these three SNPs (Kathiresan *et al.*, 2008) was confirmed by these studies. Further investigations in larger populations are now required to substantiate the hypothesis that these associations are prevalent in larger HIV+ cohorts. Such confirmation may prove useful for clinicians requiring a future predictor of HADL risk in HIV+ individuals.

These studies were unable to confirm the inverse association with HDL-c levels previously reported as a consequence of reduced SRB1 protein levels linked with the C allele in the SCARB1 SNP rs2278986C/T (West *et al.*, 2009). The investigated cohort contained 2 homozygous CC genotypes (both female), and 22 heterozygotes carrying one C allele but HDL-c levels were low for the men and women in the cohort. The studies were unable to confirm an association with this SNP in the homozygous TT patients which would have indicated an opposite effect in HIV+ populations.

An association with decreased HDL-c in patients with the T allele was confirmed when it was associated with a combination of demographic factors including age, height and the following SNPs: ABCA1 rs2230806G/A, A allele; LDLR rs1529729C/T, C allele; ABCG1 rs1893590A/C, C allele; ABCG8 rs6544713C/T, T

allele; and ApoB rs693A/G, G allele. The same combination was also confirmed as associated with elevated total cholesterol and increased LDL-c after 24 weeks on a lipid-sparing regimen of un-boosted ATV in the 24/32 patients in the HIV+ cohort. This was interesting because these were undesirable lipid profiles and an improvement in the parameters was not observed after 24 weeks of RTV absent therapy. Whilst previous studies reported an increased risk of combined SNPs in non-HIV related metabolic disorders, it is necessary to remain cautious when predicting the same outcome in larger HIV+ cohorts in the absence of further validation of these findings. If these findings are confirmed in larger populations and different ethnicities, these SNPs could form the basis of a matrix for predicting HADL outcomes with future treatment regimens.

The association the AA genotype of the ABCG1 rs1893590A/C SNP with lower HDL-c levels in women which was previously reported in HIV- patients (Abellan *et al.*) was unconfirmed as there were only 9 females in the cohort with the AA genotype and their HDL-c levels were higher than was observed in all females and the combined genders group. The absence of validation of these associations may be attributed to small numbers in the cohort, but the trend in the group was towards higher levels of HDL-c in the homozygous AA females. An association was observed with reduced baseline TG values and the C allele although again the numbers involved were low (7) and validation in a larger cohort would be required prior to reporting this as a significant association. Elevated total cholesterol and increased LDL-c levels were associated with the C allele at week 24 when the SNP was grouped with a number of demographic factors and other SNPs including: age;

height; and a combination of five SNPs: ABCA1 (rs2230806G/A, A allele); LDLR (rs1529729C/T, C allele); SCARB1 (rs2278986C/T, T allele); ABCG8 (rs6544713C/T, T allele); and ApoB (rs693A/G, G allele).

In summary, the data in thesis supports the role of LXRα as a master regulator of metabolic target genes involved in cholesterol homeostasis, bile acid metabolism and the response to RTV in a hepatic cell line. It has confirmed the role of RTV as an agonist at LXRα. It has also hypothesised the role of other metabolic NRs, including FXR, PPARα and HNF4α, in the contrasting gene expression profiles in response to ATV and RTV in the hepatic cell line. It has shown that both FXR and LXRα gene expression was simultaneously up-regulated and hypothesised that this dis-regulation could be implicated in the deleterious metabolic side effects associated with RTV. It has confirmed that the contrasting profiles of RTV and ATV are not due to higher intracellular concentrations of the former. This thesis has shown that antagonism of LXRα by chemical and biological means can alter the effects of RTV on key target genes, including SREBP1-c, previously implicated in the deleterious lipid side effects of PI therapy. This thesis has also identified areas of interest for future investigation in the complex mechanisms which regulate lipid metabolism, which include a potential therapeutic use for inhibition of LXRα by siRNA or a LXRα antagonist. Potential therapeutic inhibition of LXRα needs to be isoform specific and site directed to hepatic LXRα to facilitate normal homeostasis between cholesterol and bile acid production. The liver is the primary site of de novo cholesterol biosynthesis and enterohepatic recirculation. To underpin the findings in this thesis it would be beneficial to undertake further studies involving multiple NR knockdown

or antagonism. The use of siRNA to simultaneously knockdown LXRα and PPARα would provide the reader with an insight into the influence of LXRα/PPARα heterodimers in the response to RTV together with a test of the hypothesis that LXRα up-regulates ApoC-III via its down-regulation of PPARα. These experiments could be under-pinned by co-incubation of RTV with the LXRα antagonist 22(S)-hydroxycholesterol and the PPARα antagonist GW7647 (Tocris Bioscience) initially in the Huh7 hepatic cell line. Co-incubation of RTV and HX531 (Tocris Bioscience) an antagonist of RXRα would provide an insight as to whether the effects of RTV on LXRα and its downstream targets can be inhibited by antagonism of it permissive heterodimer partner.

This thesis has also investigated SNPs in LXRα, ABCA1, LDLR, ApoB, SCARB1, ABCG1 and ABCG8 which were previously implicated in metabolic complications in HIV- populations to determine whether there was a correlation with impaired lipid parameters in two HIV+ patient cohorts with HADL symptoms. These correlations and lack of associations were discussed in the context of the small patient cohorts involved, with the potential future clinical implications if the associations can be validated in larger HIV+ patient cohorts.

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